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Award Number: W81XWH-05-1-0012

TITLE: Identification and Characterization of Ovarian Carcinoma Peptide Epitopes Recognized by Cylotoxic T Lymphocytes

PRINCIPAL INVESTIGATOR: Kevin T. Hogan, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

REPORT DATE: November 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Nov 2008	Final	1 Nov 2004 – 31 Oct 2008

4. TITLE AND SUBTITLE

Identification and Characterization of Ovarian Carcinoma Peptide Epitopes Recognized by Cylotoxic T Lymphocytes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0012

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kevin T. Hogan, Ph.D.

E-Mail: kh6s@virginia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia
Charlottesville, VA 22904

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of the research was to identify new ovarian cancer tumor antigens that could be used in the development of an ovarian cancer vaccine. The scope of the work involved identifying peptide antigens recognized by ovarian cancer reactive cytotoxic T lymphocytes (CTL). Eleven ovarian cancer cell lines were characterized for their expression of tumor antigens and MHC molecules This was significant because it provided a resource that could be used for new antigen identification work. CTL were generated against autologous ovarian cancer cells but no evidence was found for the recognition of shared antigens which is significant because this is the type of antigen needed for vaccine development. New methodology was devised for the efficient assessment of the immunogenicity of peptides derived from known protein antigens. This work was significant because it resulted in the identification of two new HLA-A2-restricted antigens derived from TAG-1. It was demonstrated that the treatment of ovarian cancer cells with a demethylating agent is capable of turning on the expression of a large number of cancer/testis antigens and increasing the expression of class I MHC molecules which is significant because known antigens can now be used to target cells that would otherwise escape recognition by CTL.

15. SUBJECT TERMS
Ovarian carcinoma, immunotherapy, cytotoxic T lymphocytes, epitope, peptide, major histocompatibility complex (MHC)-encoded molecules, class I MHC molecules, HLA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	75	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The *subject* of this research is the identification of ovarian cancer antigens that are recognized by cytotoxic T lymphocytes (CTL). The *purpose* of the research is to identify ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. Specifically, we are attempting to identify peptide antigens that associate with class I major histocompatibility complex (MHC) coded molecules, and which are capable of stimulating an ovarian cancer cell reactive CTL response. The *scope* of this work involves (1) identifying the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian cancer cell reactive CTL.

BODY

1. Identify the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification (Months 1-36)

1.1. Establish a panel of ovarian cancer cell tumor lines. (Months 1-30)

Ten previously established ovarian cancer lines including CAOV-3 (1), CAOV-4 (2), COV413 (3), ES-2 (4), OV-90 (5), OVCAR-3 (6), SK-OV-3 (7), SW626 (8), TOV-21G (5), and TOV-112D (5) and one newly established ovarian cancer line (TTB-6) were used in this study. When establishing new ovarian cancer cell lines it is important to determine that the line is of epithelial origin and not fibroblast origin, as the latter cell type can readily become established in a culture initially containing both cell types as is usually the case with patient samples. One characteristic of epithelial cells that can be used to distinguish them from other cell types is the expression of cytokeratins (9). The mAb CAM5.2 (10) which recognizes cytokeratins 7 and 8 (Ck 7/8) (11), and the mAb NCL-5D3 (12) which recognizes cytokeratin 8, and to a lesser extent cytokeratins 18 and 19 (Ck 8/18/19), have been shown to recognize ovarian cancer cells (5, 10, 12, 13). In contrast, mAb AS02 recognizes CD90 on the surface of fibroblasts (14).

To confirm that TTB-6 was epithelial in origin, each of the lines was tested in flow cytometry for reactivity with mAb NCL-5D3 (anti-Ck 8/18) as an epithelial marker and mAb AS02 (anti-CD90) as a fibroblast marker. OVCAR-3, a well-studied ovarian cancer line was positive for Ck 8/18 expression and negative for CD90 expression, while CCD39SK, a skin fibroblast line obtained from the ATCC, demonstrated the opposite pattern of expression (Appendix 1, Fig. 1). TTB-6 was positive for Ck 8/18 expression and negative for CD90 expression (Appendix 1, Fig. 1), thus confirming the epithelial origin of the cell line. With the exception of ES-2 and TOV-112D, the remaining ovarian cancer lines were also Ck 8/18 positive and CD90 negative (data not shown). ES-2 did not express Ck 8/18, and greater than 90% of the cells were negative for CD90 (Appendix 1, Fig. 1). TOV-112D did not express Ck 8/18, but did express CD90 (the small, CD90 negative population was present in two of five experiments).

In contrast to the other nine ovarian cancer cell lines tested here, both ES-2 and TOV-112D were not recognized by mAb NCL-5D3 (Ck 8/18/19 specific) (Appendix 1, Fig. 1). TOV-112D was previously shown to be recognized by mAb CAM5.2 (Ck 7/8 specific) (5), which in combination with the present results, indicates the line expresses Ck 7, but not Ck 8/18/19. ES-2 was previously shown to be recognized by mAb OV-TL 12/30 (Ck 7 specific) but not by an antibody specific for Ck 8 (13), which in combination with the results presented here, indicates that the line expresses Ck 7 and not Ck 8/18/19. These results indicate that not all ovarian cancer cell lines are uniform in their expression of particular cytokeratins, and that multiple antibodies may be needed to accurately determine if a particular cell line expresses one or more cytokeratins.

The recognition of CD90 on TOV-112D by mAb AS02 suggests that the line is a fibroblast (Appendix 1, Fig. 1), however, several lines of evidence argue against this interpretation. First, it has previously been demonstrated that mAb CAM5.2 binds to TOV-112D (5). As indicated above, this result in combination with our own indicates that the cells express CK 7 associated with epithelial cells and not fibroblast. Second, the line expresses eight tumor antigens (see below), the expression of which is associated with tumor cells and not fibroblasts. Third, even if the small, CD90 negative population in TOV-112D represented epithelial cells and the large, CD90 positive population in TOV-112D represented fibroblasts, this could not be reconciled with the flow cytometry data. In these experiments, the entire TOV-112D population of cells is uniformly Her2/neu positive and MAGE-A1 positive, and the positive populations are clearly separated from the negative control. Thus, expression of Her2/neu and MAGE-A1 cannot be accounted for by a small, sub-population of cells, but rather reflects expression by all the cells in the population. Fourth, the small CD90 negative population was randomly observed in only two of five experiments, thus arguing against this population accounting for the expression of the tumor antigens. Taken as a whole, these results argue that TOV-112D is of epithelial origin, despite the fact that it expresses CD90. As mAb AS02 has been used in conjunction with magnetic beads to deplete cell cultures of fibroblasts (15), caution must be used to first ensure that the epithelial cell population does not also co-express CD90. It is difficult to accurately estimate how frequently ovarian cancer cell lines might express CD90 as the 95% confidence interval for the frequency based on a measurement of one positive line among eleven lines is 0.2 to 41.3%.

In order for the ovarian cancer lines to be useful in studies designed to determine the specificity of tumor reactive CTL it is necessary to know which class I MHC molecules the lines express. This question was addressed by a two-fold approach. First, the class I MHC genotype of each of the ovarian cancer lines was determined by PCR analysis (Appendix 1, Table 2). The results of the PCR typing indicate that a minimum of two of the lines (COV413 and SW626) and perhaps an additional two lines (CAOV-3 and CAOV-4) are either homozygous for expression of the HLA-A, -B, and –C alleles, or that they have undergone the deletion of a complete haplotype on one copy of chromosome 6. As the loss of class I and class II MHC expression through chromosomal deletions is a relatively frequent event in cancer cells (16), the loss of a haplotype is a likely explanation for this observation. Homozygous expression cannot be excluded, however, as typing of normal cells from the corresponding patients would be required and such material is not available.

Second, because tumor cells frequently lose the expression of MHC molecules through a variety of mechanisms (16), we also sought to determine if class I MHC molecules could be detected on the surface of the cell lines. mAb W6/32, specific for an epitope present on all class I MHC molecules was used for the analysis (Appendix 1, Fig. 2). Each of the lines was positive for class I MHC expression, albeit at levels that are low to moderate in comparison to the B-LCL, JY, which expresses high levels of class I MHC molecules. This information is particularly informative when choosing ovarian cancer lines for use as stimulators or targets when stimulating or assessing the specificity of ovarian cancer-specific CTL.

In the same analysis we also sought to determine if ovarian cancer cells express class II MHC molecules as determined by their ability to bind the class II MHC-specific mAb, L243 (Appendix 1, Fig. 2). Most lines do not express class II MHC molecules, although low expression was detected on CAOV-3, CAOV-4, OVCAR-3, SW626, and a subpopulation of ES-2. This indicates that these cell lines may have the ability to stimulate class II MHC restricted responses.

mAbs specific for some of the more prevalent class I MHC molecules in the population are available and were used to assess the expression of individual class I MHC molecules on the ovarian cancer lines (Appendix 1, Table 3). Based on the genotype of the cells, each of the

HLA-A2, -A3, -A68, -A69, and -B7 molecules were generally expressed at low to moderate levels in comparison to expression on C1R-A2, C1R-A3, C1R-B7, and JY. Expression of HLA-A2 and/or HLA-B7 on OV-90 and OVCAR-3 was particularly low, while expression of HLA-A3 and HLA-B7 on SW626 was quite high.

To be of value in defining the antigens recognized by ovarian cancer-specific CTL it is also important to have a panel of tumor cell lines that have been characterized for antigen expression. The eleven ovarian cancer cell lines studied here were tested for the expression of twelve cancer/testis antigens (CTA), Her-2/neu, and FBP. PCR antigen-specific primers (Appendix 1, Table 1) was used to determine the mRNA expression levels of these fourteen tumor antigens. Each ovarian cancer line had a unique pattern of tumor antigen expression, and expressed between six and twelve of the tested antigens (Appendix 1, Table 4; Appendix 2, Table 1). The expression of the individual antigens among the cancer lines ranged from two to eleven positive lines for each of the antigens. The variability in expression occurred within the cancer/testis antigens, while Her-2/neu and FBP were found to be expressed in each line tested.

The availability of antibodies to some of the tested antigens allowed for the further assessment of the antigens at the protein level (Appendix 1, Fig. 3). Her-2/neu was clearly over-expressed in SK-OV-3 (100.2-fold over background), and is present at 3.0- to 7.4-fold over background in all the remaining lines with the exception of ES-2. These results are consistent with a previous report demonstrating that SK-OV-3, TOV-21G, and TOV-112D express Her-2/neu as demonstrated by immunohistochemistry (5). Likewise, FBP was clearly over-expressed at the protein level in CAOV-3 (14.1-fold), OV90 (17.5-fold), SW626 (10.1-fold), and TTB-6 (9.2-fold), and to a lesser extent in CAOV-4 (4.9-fold), OVCAR-3 (3.2-fold), and SK-OV-3 (4.0-fold). As with Her-2/neu, a positive PCR at 30 and 40 cycles was poorly predictive of total protein. These results indicate that caution must be used when assessing antigen expression solely on the basis of the strength of the PCR signal. The lack of a strong correlation between PCR reactivity and antibody reactivity could be due to the fact that relatively high mRNA expression saturates the PCR signal even at 30 cycles of amplification, gene-specific mutations preclude the ability of the proteins to be expressed, or that additional factors regulate protein expression.

The results show that the anti-MAGE-A1 antibody bound to three lines (COV413, TOV-21G, and TOV-112D) that were PCR negative for the MAGE-A1 gene (Appendix 1, Table 4 & Fig. 3). The most likely explanation for binding to MAGE-A1 negative cell lines is cross-reactive binding on other MAGE-A proteins as has been reported for other MAGE-specific antibodies including 57B and 6C1 (17). An analysis of the results does not readily indicate another MAGE-A gene product that might be recognized. As we have used PCR to only test for the seven most prevalent of the eleven expressed MAGE-A genes, the possibility remains that additional, less prevalent MAGE-A genes are expressed in the cell lines and recognized by the antibody. It is also possible that only a small fraction of a line expresses a particular gene when that line is found to be positive by PCR, and that the antibody binding results are an accurate assessment of protein expression for those lines.

As CEA expression can be detected by immunohistochemistry in a low percentage of ovarian cancer samples (18), each of the ovarian cancer cell lines was also tested for CEA expression. As determined by flow cytometry, only OV-90 expressed CEA. Interestingly, SW626 did not express CEA. Although SW626 was originally reported to be an ovarian cancer cell line (8), a more recent report provides evidence that it may actually be of colonic origin (19). The expression of FBP and the lack of expression of CEA is, however, consistent with the line being of ovarian origin.

Both primary and cultured ovarian cancer cells have been shown to express TGF-β (20-23). TGF-β inhibits the *in vitro* generation of CTL (24-26) and blocks *in vivo* tumor immunosurveillance (27). To the extent that cell lines in our ovarian cancer cell line panel will be used in an attempt to stimulate ovarian-specific CTL, it is important to know whether or not any of the ovarian cancer cell lines in our panel express suppressive cytokines.

The production of TGF-β1, TGF-β2, TGF-β3, and IL-10 was measured in supernatants collected from the ovarian cancer lines following 48 h of growth (Appendix 1, Fig. 4). TGF-β exists in two forms, active and latent. Active TGF-β can be measured directly by ELISA, while the latent form must first be activated, and was done here by acid treatment. Total TGF-β is thus a measure of both pre-existing, active TGF-β, and newly activated TGF-β derived from the latent form of the protein.

Only low amounts of active TGF-β1 were measured in CAOV-3, COV413, and ES-2. Following acid activation, the total TGF-β1 measured from these same three cell lines was elevated above that endogenously present in FBS. The remaining ovarian cancer lines either did not produce, or only produced negligible amounts of TGF-β1. Active TGF-β2 was measured in all of the supernatants obtained from the ovarian cancer cell lines and ranged from about 15-100 pg/ml above that found in FBS. Substantial amounts of total TGF-β2 (>1,500 pg/ml) were found in COV413, OVCAR-3, SW626, and TOV-112D-derived supernatants, while lesser amounts (>300 pg/ml) were found in ES-2, OV-90, and SK-OV-3. Active and total TGF-β3 was either absent or present in only small amounts (<25 pg/ml). These concentrations may be biologically significant as TGF-β1 and –β2 at concentrations greater than about 500 ng/ml have been shown to suppress the *in vitro* generation of CTL (24-26, 28).

IL-10 is infrequently expressed in ovarian cancer cell lines (23, 29, 30), is present in the ascites of patients with ovarian cancer (30, 31), and is associated with the suppression of T cell responses (32). Only one ovarian cancer cell line (SW626) of the eleven tested here expressed appreciable amounts of IL-10 (Appendix 1, Fig. 4), an amount that was previously shown to be biologically significant in blocking anti-CD3-induced T cell proliferation (33).

The ovarian cancer cell lines characterized here all express class I MHC molecules and a variety of tumor antigens. Some, but not all of the lines, also express immunosuppressive cytokines. This comprehensive analysis will serve to increase the utility of these cell lines in the characterization of antigens recognized by ovarian cancer-specific CTL.

The characterization of TAG antigen expression was further extended to uncultured ovarian cancer cells obtained from patients. The percentage of tumors expressing TAG ranged from a high of 35% (TAG-1) to a low of 9% (TAG-2b, TAG-2c) in 23 tested samples (Appendix 2, Table 2). These results indicate that TAG expression is not an artifact related to in vitro culture conditions, and further indicates that the antigens are expressed at levels within the population that are comparable to other cancer-testis antigens.

1.2. Establish class I MHC-restricted, ovarian cancer cell reactive CTL lines. (Months 1-30)

The goal of this section was to generate CTL lines which recognize ovarian cancer tumor cells. Patient derived ascites was used as the source of tumor associated lymphocytes (TAL) and autologous tumor cells. The lymphocytes were generally stimulated four times with autologous tumor before testing in ^{51}Cr-release assays for their ability to kill autologous tumor, class I MHC-matched tumor, and peptide-pulsed, B-LCL. The peptides used for these studies were those synthesized for Specific Aim 2 (Table 1). The peptides were tested based on the premise that if any of the peptides are actually presented by tumor cells, then T cells specific for those peptides should be generated when the T cell stimulation is performed with tumor. The following is a summary of our efforts to generate such ovarian cancer specific CTL:

TAL546 (HLA-A3, -B7). This CTL initially showed weak reactivity to the ovarian cancer cell line ES-2. In an attempt to stimulate the population of lymphocytes with this specificity the TAL were subsequently stimulated twice with ES-2 and then tested for their ability to kill ES-2 and additional HLA-A3$^+$ ovarian cancer cell lines (Table 2). The data indicate that stimulation with ES-2 tumor cells has not preferentially stimulated an ES-2-specific response, nor a response that recognizes any other HLA-A3 expressing tumor. These T cells were also tested for reactivity to the HLA-A3 and HLA-B7 restricted, FBP, HER-2/neu, and mesothelin-derived peptides listed in Table 11. None of the peptides was recognized.

TAL572 (HLA-A2). This CTL initially showed reactivity to autologous tumor and possibly the allogeneic ovarian cancer tumor line SKOV3-A2 (Table 3). Antibody blocking experiments with antibodies directed towards class I MHC molecules were used to determine if an HLA-A2-restricted antigen was being recognized. These experiments demonstrated little to no blocking (data not shown) suggesting that the response is not HLA-A2 restricted. Cold target inhibition experiments were also performed with both “hot” TAL572 and SKOV3-A2, but reproducible data demonstrating that a shared, HLA-A2-restricted antigen was being recognized could not be obtained (data not shown). These T cells also did not recognize any of the HLA-A2-restricted, FBP or mesothelin-derived peptides listed in Table 1 (data not shown).

TAL11770 (HLA-A2). In a modification of our standard stimulation protocol, the lymphocytes first had the CD25$^+$ population removed by immunomagnetic separation so as to remove any CD4$^+$CD25$^+$ T regulatory cells that might be present and which could limit the CTL response to the autologous tumor. The remaining T cells were then stimulated with autologous tumor. TAL11770 initially showed weak reactivity on autologous tumor, however, with repeated stimulations this reactivity was lost and no reactivity was seen on HLA-A2 matched ovarian tumors (Table 4). These results indicate TAL11770 does not have any tumor reactivity. Testing against the HLA-A2-restricted peptides derived from FBP and mesothelin (listed in Table 1) did not reveal any peptide-specific reactivity.

TAL0319 (HLA-A2). The TAL0319 CTL demonstrated non-specific killing against both HLA-A2 positive and negative ovarian cancer lines, while at the same time losing reactivity against the autologous tumor (Table 5). The T cell line is thus not useful for antigen identification. The line was also tested against C1R-A2 pulsed with the HLA-A2 peptides listed in Table 1. The results demonstrated that the T cells had high non-specific reactivity against the C1R-A2 line alone, with no evidence of peptide-specific recognition (Table 6).

TAL0406 (HLA-A1, -A3). The TAL0406 CTL demonstrated non-specific killing against both HLA-A3 positive and negative ovarian cancer lines, while only demonstrating weak killing against autologous tumor cells (Table 7). The line was subsequently stimulated with either ES-2 or TOV-112D in order to preferentially stimulate CTL specific for one of these tumors. Under these conditions the T cells eventually became non- to poorly-reactive. These characteristics mean that the line cannot be used for antigen identification. The T cells were further tested against C1R-A1 and C1R-A3 pulsed with the respective HLA-A1 and HLA-A3 restricted peptides listed in Table 1. The analysis indicated that the T cell line had a high level of non-specific reactivity against C1R-A1 and C1R-A3 in the absence of peptide, and did not detect any peptide-specific reactivity against any of the tested peptides (Tables 8, 9).

TAL0511 (HLA-A2, -A3). Following four initial stimulations with autologous tumor the T cells had a high level of non-specific reactivity that was observed on both HLA-matched and unmatched targets (data not shown). Continued stimulation with autologous tumor ultimately resulted in a T cell line that did not recognize either HLA-matched allogeneic ovarian cancer cell lines or the autologous tumor (Table 10). The lack of killing of any tumor target precludes the ability to use these TAL for antigen identification. The T cells were also tested to determine if they recognized any of the HLA-A2 or HLA-3 restricted peptides listed in Table 1. Although TAL0511 exhibited

relatively low background killing of C1R-A2 and C1R-A3, no specific reactivity could be detected against any of the tested peptides (Tables 11, 12).

TAL0724. The patient from whom these cells were derived was HLA-typed as HLA-A24,32; B35,40). These alleles were not among those which we had initially targeted because they are not expressed by an appreciable percentage of the population. Nonetheless, we attempted to grow tumor specific T cells from this patient so as to gain additional experience that might prove useful in expanding ovarian cancer specific CTL. After four stimulations these T cells did not recognize autologous tumor and demonstrated a high level of non-specific reactivity against allogeneic tumors (data not shown). Therefore, we did not pursue the use of these T cells any further.

TAL567 (HLA-A2). TAL567 CTL recognize the autologous tumor from the sample used to derive the CTL line (TPF567) and they also recognize autologous tumor derived from a second ascites sample from the same patient (TPF568) (Table 13). Unfortunately, the CTL do not recognize any of the HLA-A2 matched ovarian cancer cell lines, thus indicating that the CTL either recognize an antigen unique to the autologous tumor, or at most they recognize an antigen that is not prevalently shared among different HLA-A2 expressing tumors. It has also not been possible to establish a tumor line from either TPF567 or TPF568. Taken together, these results indicate that these CTL are not good candidates for antigen identification. The CTL were also tested against the HLA-A2 restricted, mesothelin-derived peptides listed in Table 1, but no reactivity was observed in response to any of them (Table 14).

TAL519 (HLA-A1, -A2, -B7). In a modification of our standard stimulation protocol, the lymphocytes first had the $CD25^+$ population removed by immunomagnetic separation so as to remove any $CD4^+CD25^+$ T regulatory cells that might be present and which could limit the CTL response to the autologous tumor. The remaining T cells were then stimulated a total of four times with autologous tumor. When tested against HLA-A2 or HLA-B7 matched allogeneic ovarian cancer cells or against autologous tumor, the T cells had a relatively low amount of non-specific reactivity on non-HLA-matched target cells, but no greater reactivity was observed on the matched targets (Table 15). Thus, the T cells did not appear to have developed any specific tumor reactivity. The T cells were further tested to determine if they recognize any of the HLA-A1, -A2, or –B7 restricted peptides from FBP, HER-2/neu, mesothelin, and TAG (Table 1), and none were found to be recognized.

The reactivity of the generated T cell lines can broadly be categorized into three groups. Half of the lines fell into the first group which includes those lines for which reactivity could not be detected on either the autologous tumor or on HLA-matched, allogeneic ovarian cancer tumors (Table 2, 4, 10, 15). Whether the lack of reactivity is reflective of a lack of immunogenicity or an over-riding effect of an immunosuppressive environment hasn't been established at this time. The second group includes those lines with a substantial amount of reactivity against both autologous tumor and HLA-matched, allogeneic ovarian cancer tumors (Table 5, 7). This "non-specific" reactivity cannot be simply ascribed to natural killer cell activity as the target cells all express varying amounts of class I MHC molecules and should generally be resistant to natural killer cell mediated cytotoxicity. Finally, two of the lines exhibited specific cytotoxicity against autologous tumor cells (Table 3, 13). TAL572 recognized autologous and also demonstrated reactivity against the HLA-A2 matched SK-OV3.A2 ovarian cancer cell line. Although this suggested that an HLA-A2-restricted antigen was being recognized, this could not be confirmed in antibody blocking experiments. Thus, the most likely interpretation of the results is that the T cells were recognizing an antigen unique to the autologous tumor cells. Similarly, TAL567 T cells only recognized autologous tumor, again suggesting that they recognize a unique, rather than shared tumor antigen. While identifying unique antigens may be useful from an

individualized therapy standpoint, it is not useful in the development of a vaccine that is useful for the treatment of most ovarian cancer patients.

1.3. Identify the peptide antigens recognized by the CTL established in 1.2. Each identification project will last an average of 4-6 months, and a given antigen identification project is expected to yield from one to several new peptide antigens. (Months 6-36)

The antigens recognized by cancer-reactive CTL have been broadly categorized as either being unique or shared antigens. Unique antigens are expressed only on the tumor of a particular patient and are not found on tumors obtained from other patients. Conversely, shared antigens are found on the tumors obtained from multiple patients. From the perspective of developing a vaccine for the treatment of ovarian cancer, the identification of shared antigens is the goal of this sub-aim. As indicated in 1.2 above, we have not yet been able to identify ovarian reactive CTL that recognize a shared antigen which has prevented us from pursuing this objective.

In lieu of identifying new antigens, it would be equally advantageous if the utility of existing antigens could be expanded. For example, a large number of cancer/testis antigens have been identified, but because they are not expressed on ovarian cancer cells from all patients, a vaccine based on only one or a few of them will necessarily be ineffective in a large fraction of ovarian cancer patients (34). Cancer/testis antigen expression is regulated at the transcriptional level by promoter methylation, and treatment of cells with the demethylating agent 5-aza-2'-deoxycytidine (DAC) leads to the upregulation of cancer/testis antigens in a variety of cancers (See Appendix 3 for references). The expression of class I MHC molecules is also regulated by promoter methylation, and treatment of tumors with DAC can frequently upregulate class I MHC expression (See Appendix 3 for references). We therefore asked if the treatment of ovarian cancer cells with DAC would increase the number of cancer/testis antigens expressed per cell line, and if the treatment would increase class I MHC molecule expression on those cell lines with poor class I MHC expression.

Based on the fact that most studies designed to investigate the effect of DAC treatment on the expression of CTA genes and class I MHC proteins have treated cells for 2-4 days with 1-2 μM DAC (35-39), we treated 11 ovarian cancer cell lines with 1 μM DAC for 3 days. CTA gene expression was assessed by performing 30 and 40 cycles of PCR analysis. PCR with primers for the housekeeping gene GAPDH was first used to ensure that amplifiable cDNA had been obtained from each cell line. The results of this analysis demonstrated that all of the ovarian cancer cell lines had comparable levels of GAPDH gene expression (data not shown). PCR amplifications were then performed for 12 CTA genes (Appendix 3, Table 1; Appendix 3, Supplementary Fig. 1). DAC treatment resulted in higher levels of expression of at least 1 CTA gene in 10 of the 11 cell lines. Most cell lines had increased levels of expression of 1 to 5 CTA genes, while 1 cell line had increased levels of expression of 7 CTA genes. The expression levels of the MAGE-A1 and NY-ESO-1 genes were most frequently enhanced while the MAGE-A2, MAGE-A6 and MAGE-A12 genes were not enhanced in any of the cell lines tested. The expression of the remaining CTA genes was shown to increase in 1 to 3 cell lines each.

Class I MHC protein expression in the untreated and DAC treated cells was assessed by flow cytometry using mAb W6/32, which recognizes an epitope common to all class I MHC molecules, and mAbs to the individual class I MHC molecules HLA-A2 (mAb CR11-351), HLA-A3 (mAb GAP-A3), and HLA-B7 (mAb ME1-1.2). The treatment resulted in a reproducible, but small increase in HLA-A2 and total class I MHC expression in OV-90 (Appendix 3, Supplementary Fig. 2). Conversely, the treatment of SK-OV-3 with DAC did not reproducibly increase HLA-A2, HLA-A3, or total class I MHC expression (Appendix 3, Supplementary Fig. 2). The results obtained with OV-90 and SK-OV-3 were representative of those obtained with the remaining 9 ovarian cancer cell lines (data not shown).

The effect of varying the length of treatment time with DAC was studied by comparing CTA gene and class I MHC protein expression levels in selected cell lines (ES-2, OV-90, OVCAR-3, and SK-OV-3) treated with 1 µM DAC for 3, 5, and 7 days. GAPDH gene expression in the 4 cell lines was not affected by any length of treatment (Appendix 3, Fig. 1). MAGE-A1 gene expression was highest at day 7 for 3 of the 4 cell lines, and NY-ESO-1 gene expression was highest at day 7 for all 4 cell lines. Similar results were observed when class I MHC molecule expression was measured by flow cytometry (Appendix 3, Fig. 2 and data not shown). The highest levels of expression of HLA-A2, HLA-B7, and overall class I MHC molecule expression on OVCAR-3 were observed on day 7 following treatment. For SK-OV-3, expression of HLA-A2, HLA-A3, and overall class I MHC expression was increased to similar levels following 5 and 7 days of treatment, and in all cases exceeded that observed following 3 days of treatment. Consequently, subsequent experiments were conducted with 7 days of DAC treatment.

The optimal concentration of DAC needed to enhance the expression of CTA genes and class I MHC proteins was next determined. Ovarian cancer cell lines were untreated, or treated with varying concentrations of DAC (0.1 to 30 µM) for 7 days. PCR analysis of OVCAR-3 and SK-OV-3 showed that the treatment did not affect GAPDH gene expression (Appendix 3, Fig. 3). Conversely, the expression of CTA genes was enhanced by increasing concentrations of DAC, with maximal expression requiring a minimal concentration of 1 – 3 µM. Maximal expression of class I MHC molecules required a higher concentration of DAC, with 10 µM appearing to be optimal (Appendix 3, Fig. 4). Based on the results of dose response and time course experiments, 7 days of incubation with 10 µM DAC was adopted as the standard treatment to enhance CTA gene expression and class I MHC protein expression.

Having determined the optimal concentration and incubation period for DAC treatment, we next treated 11 ovarian cancer cell lines with 10 µM DAC for seven days. CTA gene expression was determined with both 30 and 40 cycles of PCR analysis. Results of a single experiment analyzed with 30 cycles of PCR demonstrate several points (Appendix 3, Fig. 5). First, CTA genes are not readily detectable in most untreated cell lines when tested with 30 cycles of PCR amplification. ES-2 and OV-90 are exceptions in that many of the CTA genes can be detected in these cell lines without prior exposure to DAC. Second, treatment with DAC can be very effective in upregulating the expression of some CTA genes (MAGE-A1, MAGE-A3, MAGE-A10, NY-ESO-1) while it has little (MAGE-A4) to no (MAGE-A2) effect on the expression of other CTA genes when measured at 30 cycles of PCR. Third, the increased expression of a particular CTA gene in one cell line does not predict that it will be increased in all cell lines (MAGE-A6, NY-ESO-1). Fourth, the increased expression of a particular CTA gene in 1 cell line is not predictive of other CTA genes being increased in the same cell line.

The sensitivity of this analysis was increased by also conducting the CTA gene expression measurements with 40 cycles of PCR amplification. The combined results of 30 and 40 cycles of PCR analysis is presented in Appendix 3, Table 1 and Appendix 3, Supplementary Fig. 3. The number of CTA genes with enhanced expression ranged from 1 to 11 per cell line, with most cell lines having enhanced expression of 6 to 11 CTA genes. Correspondingly, the expression of 9 of the 12 CTA genes were enhanced in at least 7 different ovarian cell lines. Expression of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, and NY-ESO-1 was increased in every evaluable cell line. Only MAGE-A2 did not increase in any cell line, and in fact, tended to decrease from low to no expression following treatment (Appendix 3, Supplementary Fig. 3).

The expression of the MAGE-A1 protein was measured to determine if increased CTA gene expression levels also lead to an increase in the expression of the corresponding protein. Although many lines showed a large increase in the expression of the MAGE-A1 gene when treated with 10 µM DAC for 7 days (Table 1, Supplementary Fig. 3), the cell lines did not show a correspondingly large increase in protein expression (Appendix 3, Fig. 6). MAGE-A1 protein

expression increased greater than 3-fold in CAOV-3 and TOV-21G, and greater than 1.9-fold in OV-90, OVCAR-3, and TOV-112D.

Class I MHC protein expression was also assessed following treatment of the 11 ovarian cancer cell lines with 10 µM DAC for 7 days (Appendix 3, Fig. 7). Overall, two patterns of expression emerged. When class I MHC molecules are already expressed at high levels (ex. CAOV-3 and CAOV-4) there was no to only a modest enhancement of expression. When protein expression was low, increases in expression levels ranged from about 2 to 10-fold, however, despite the high relative increase in expression, the overall protein expression levels remained below that found in a cell lines naturally expressing high levels of class I MHC molecules.

The results presented here clearly indicate that the treatment of ovarian cancer cells with DAC can lead to an enhancement in expression of CTA genes (Appendix 3, Table 1; Appendix 3, Supplementary Fig. 3). In comparison to other studies in which 3 to 4 days of treatment with 1-2 µM DAC was routinely used to upregulate CTA gene expression (35-39), ovarian cancer cell lines required 7 days of treatment with DAC at 1-3 µM to achieve the highest levels of CTA gene expression (Appendix 3, Fig. 1, 3). The need for a lengthier treatment time may reflect the longer doubling times that ovarian cell lines have in comparison to melanoma cell lines (unpublished observations). Because DAC acts by inhibiting methylation of a newly synthesized DNA strand, it would be expected that the rate at which demethylation will occur would be a function of the replicative rate of the cells.

It is important to note, however, that DAC treatment is not a universal panacea for upregulating CTA gene expression. MAGE-A2 gene expression, for example, does not increase, but rather decreases in expression following DAC treatment (Appendix 3, Supplementary Fig. 3). This result stands in contrast to the results obtained with CTA genes such as MAGE-A1, MAGE-A3, MAGE-A10, and NY-ESO-1, which when ordinarily expressed at low levels undergo a dramatic upregulation following DAC treatment. It is also evident that there is heterogeneity in the response to DAC treatment, both with respect to how frequently the expression of a particular CTA gene is upregulated in different cell lines, and with respect to how many CTA genes are upregulated in a particular cell line. Such heterogeneous response patterns of CTA gene expression are not unique to ovarian cancer cell lines, but have been demonstrated in other cancers (36, 40-42). These findings likely reflect the multiple control mechanisms that can affect the expression of any particular gene, as well as the fact that promoter demethylation will have little functional consequence if the gene in question is no longer functional due to a genetic defect.

It is not increased CTA gene expression per se that leads to increased recognition of tumor cells by CTA-specific T cells, but rather it is increased peptide production which can occur through a combination of increased protein production and/or increased protein degradation. There are few CTA-specific antibodies available to measure protein expression levels, although a number of antibodies have been developed which recognize MAGE-A proteins. Although most of the antibodies with specificity for MAGE-A proteins recognize multiple members of the MAGE-A family, the mAb 3F257 is MAGE-A1 specific (USB technical data sheet). mAb 3F257 was used to ask if MAGE-A1 protein expression increased in parallel with CTA gene expression following DAC treatment. Although MAGE-A1 protein expression increased by 1.9 to 4.0-fold in 5 of the 11 cell lines tested (Appendix 3, Fig. 6), the magnitude of the increase is relatively small in comparison to the corresponding increase in gene expression (Appendix 3, Table 1; Appendix 3, Supplementary Fig. 3). This would suggest that other factors limit the translation of MAGE-A1 mRNA, such that there is not a linear relationship between mRNA and protein expression. Alternatively, increased levels of the protein may be associated with an increased rate of degradation of the protein.

The treatment of ovarian cancer cell lines with DAC leads to an increase in the levels of class I MHC protein expression (Appendix 3, Fig. 7), similar in magnitude to that observed when melanoma cell lines are treated with DAC (37, 43, 44). When cell lines already express class I MHC molecules at intermediate to high levels, the amount of increase if any, is less than two-fold (e.g. CAOV3, CAOV4, SW626). This is in accord with the fact that when gene expression levels are already high it is likely that the promoter region of the gene already exists in an unmethylated state and treatment with DAC would not be expected to have a large effect on expression. When cell lines naturally express low levels of class I MHC molecules, treatment with DAC can increase expression by four to ten-fold (see OVCAR-3, TOV-21G, TOV-112D). In some poorly expressing cell lines such as ES-2 and SK-OV-3, however, class I MHC protein expression is increased by less than two-fold. That the expression levels in these cell lines aren't increased to the high levels naturally expressed in other cell lines likely reflects the fact that the low expression levels are only partially regulated, or are not regulated at all, by a methylation dependent mechanism. One or more genetic defects, either in the class I MHC genes themselves or in one of the ancillary genes (β_2-microglobulin, proteasomes, TAP, etc.) required for class I MHC protein expression could also account for the low expression levels of the class I MHC proteins (16) .

To determine if enhanced expression of CTA and class I MHC molecules leads to increased recognition of the treated cells by T lymphocytes, antigen-reactive $CD8^+$ T lymphocytes that recognize peptide antigens derived from the MAGE-A1, MAGE-A10, and NY-ESO-1 proteins were tested in an interferon-γ ELISpot assay for their ability to recognize DAC-treated and untreated cells. $CD8^+$ T lymphocytes that recognize the NY-ESO-1-derived peptide ASG in association with HLA-A3 (Appendix 3, Fig. 8A,C) were tested against SK-OV-3, SW626, and TOV-112D (all NY-ESO-1^+, HLA-$A3^+$) as potential positive stimulator cells and COV413 (NY-ESO-1^+, HLA-$A3^-$) as a control cell line (Appendix 3, Fig. 8B,D). DAC treatment of SW626, but not the remaining cell lines, led to an increase in T cell responses to those tumor cells. $CD8^+$ T lymphocytes that recognize the MAGE-A1-derived peptide SLF in association with HLA-A3 (Appendix 3, Fig. 8E,G) were tested against the same cell lines, all of which also express MAGE-$A1^+$ (Appendix 3, Fig. 8F,H). Treatment of SW626 with DAC was again shown to lead to an increase in the ability of the cell line to trigger interferon-γ release by the antigen-specific $CD8^+$ T lymphocytes. Finally, $CD8^+$ T lymphocytes that recognize the MAGE-A10-derived peptide GLY in association with HLA-A2 (Appendix 3, Fig. 8I) were tested for interferon-γ release against CAOV-4, COV413, and OVCAR-3 (all MAGE-$A10^+$, HLA-$A2^+$) as potential stimulators and TOV-112D (MAGE-$A10^+$, HLA-$A2^-$) as a control cell line (Appendix 3, Fig. 8J). Treatment of these cell lines with DAC did not significantly change their ability to stimulate antigen-specific $CD8^+$ T lymphocytes.

The ability of DAC treatment to functionally modify antigen expression was also tested by treating ovarian cancer cells with DAC and then determining if they were recognized by antigen specific CTL in a cytotoxicity experiment (Table 16). The ovarian cancer cell line COV413 ordinarily does not express MAGE-A10, but following treatment with DAC expresses the cancer/testis antigen at high amounts (Appendix 3, Supplementary Fig. 3). This increase in expression is sufficient to render the cells sensitive to lysis by the MAGE-A10 peptide (GLY)-specific, HLA-A2-restricted CTL line VMM528 (Table 16). A similar result was also obtained with the ovarian cancer cell line OVCAR3 (Table 16), except that the effect is likely mediated through an increase in HLA-A2 expression (Appendix 3, Fig. 7) as the line naturally expresses high levels of MAGE-A10 (Appendix 3, Supplementary Fig. 3). As expected, DAC treatment had no effect on the ability of the CTL to recognize the TOV-112D and SW626 cell lines as both of these lines do not express HLA-A2.

The results with the ELISpot and cytotoxicity analysis are important because they validate that the increased expression levels of cancer/testis antigens and class I MHC molecules following treatment with DAC is functionally important and leads to an increase in recognition of the cells by antigen-specific CTL. These results demonstrate that with the exception of MAGE-A2, DAC treatment of ovarian cancer cells can drive increased expression of eleven different cancer/testis antigens and thus greatly increasing the utility of antigens that are already known and in clinical trials.

2.0 Identify peptide antigens within the Her-2/neu, folate binding protein, and TAG proteins that give rise to ovarian reactive CTL (Months 1-36)

2.1. Predict the Her-2/neu, folate binding protein, and TAG-derived peptides that conform to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs. Have synthesized the HLA-A1 and –A3 peptides that conform to the rules in specific aim #2. The remaining peptides will be synthesized as needed for step 2.4 below. (Months 1-2)

Each of the target proteins (Her-2/neu, folate binding protein, TAG) was analyzed with the SYFPEITHI and Parker algorithms, and peptides corresponding to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs were predicted and ranked. This was followed by a visual inspection to remove any predicted peptide that had a poor score, and to remove any that would be difficult candidates to synthesize. The lists were then merged together to give two to six peptides per antigen per class I MHC molecule (Table 1). Each of the listed peptides was then synthesized and solubilized in DMSO. Because the most cost-effective means of having these peptides was synthesized was to use a multi-peptide synthesis service which synthesizes up to 96 peptides for a single price, we also had synthesized a number of mesothelin-derived peptides as it added no additional costs to the project. Mesothelin has recently emerged as a promising ovarian cancer antigen (45-47). As was done with the Her-2/neu, FBP, and TAG proteins, the SYFPEITHI and Parker algorithms were used to predict class I MHC binding motifs and the highest ranked peptides were chosen for synthesis.

2.2. PCR isolate the cDNA for Her-2/neu and folate binding proteins, and clone the cDNA for Her-2/neu, folate binding protein, and TAG, into the plasmid pcDNA3.1. Plasmids will be transfected as needed. (Months 1-3)

The folate binding protein (FBP) gene was PCR amplified from the TOV-21G ovarian cancer cell line using a 5'-primer with an incorporated *Kpn*I restriction enzyme site and a 3'-primer with an incorporated *Eco*RI site. Both the amplification product and the pcDNA3.1 vector were double-digested with *Kpn*I and *Eco*RI. The FBP gene was then directionally ligated into the vector. Following transformation into TOP10 chemically competent *E. coli* and selection of ampicillin-resistant bacterial colonies, plasmid preparations were made from individual colonies. Forward and reverse sequencing was then done to confirm the sequence of the inserted FBP gene. Sequence comparisons were made against the published sequence for FBP (Entrez accession number X62753). One clone was obtained that was free of PCR cloning artifacts and corresponded to the published sequence. The final plasmid is capable of expressing FBP in mammalian cells under the control of the CMV promoter, and the incorporation of the neomycin resistance gene allows for the G418 selection of cells that have taken up the plasmid (Fig. 1).

Because the cloning, sequencing, transfection, and selection of multiple genes in multiple cell lines is a resource intensive endeavor we delayed pursuing this objective for the Her-2/neu and TAG genes. The Her-2/neu gene has been extensively studied by other groups and is readily available. The need to clone the TAG gene has been negated by the studies described above in section 1.3 where we demonstrated that TAG expression can be induced in cell lines by treatment with DAC (Appendix 3, Supplementary Table 3).

2.3. Stimulate TIL/TAL samples from HLA-A1$^+$ and HLA-A3$^+$ ovarian patients with ovarian peptides predicted to bind to the respective class I MHC molecules. Test the specificity of the ensuing cultures for reactivity with peptide-pulsed target cells and with ovarian cancer cells expressing the appropriate class I MHC molecule and cognate protein. Confirm peptide identity with SRM mass spectrometry. (Months 4-12)

As indicated in section 1.2 above, we established ovarian cancer reactive CTL lines by stimulating the TAL obtained from ovarian cancer patients with autologous tumor. The CTL that develop in response to autologous tumor are specific for those peptides which are naturally processed and presented by the tumor. By using these CTL lines to screen for reactivity against synthetic peptides, one eliminates the possibility of identifying a peptide that is immunogenic, but not naturally processed and presented. As indicated in section 1.2 the results of these peptides has been negative and a selection of specific results is shown below.

TAL0406 CTL were used to test HLA-A1-restricted peptides including one from FBP, three from Her-2/neu, and two from mesothelin (Table 8). Because of the high background killing on C1R-A1 without peptide, the analysis is best restricted to the results obtained at an E:T of 10:1. There is no evidence, however, that any of the tested peptides were recognized as none of the peptide-pulsed targets demonstrates a significant level of killing above that of the unpulsed targets.

TAL0406 and TAL0511 CTL were used to test HLA-A3-restricted peptides including four from FBP, two from Her-2/neu, and two from mesothelin (Tables 9, 11). TAL0406 also had high background killing of C1R-A3 without peptide, but this issue was not seen with TAL0511. The results demonstrate that none of the peptides are reproducibly recognized by the CTL.

The original plan for this section was to use ovarian cancer patient-derived lymphocytes as the responder cells and peptide-pulsed B-LCL as the stimulator cells. We also proposed an alternative methodology in which the responding lymphocytes from healthy donors were stimulated with peptide-pulsed autologous dendritic cells (DC). For two reasons, we chose to begin these experiments using the alternative methodology. First, to perform these experiments most expeditiously, it was first necessary to characterize the available patient material for class I MHC, TAG, FBP, and Her-2/neu expression as has now been done under 1.1 above. Second, we had already established methodology in the laboratory to allow for the use of healthy donor lymphocytes for antigen identification. This procedure is based on that of Lu and Celis (48) and is illustrated in Figure 2. Once the lymphocytes have been stimulated four times with peptide, they are assayed for specificity as illustrated in Figure 3.

PBMC from healthy donors were obtained through Virginia Blood Services. Initial processing was done to provide: (i) genomic DNA for class I MHC molecular typing; (ii) dendritic cells to which peptide was bound, and were then used as stimulator cells; and (iii) lymphocytes which were used as the source of CD8$^+$ responder T cells. The cells were cryopreserved until an average of six donors could be simultaneously tested as described above for their response to a defined peptide/class I MHC molecule combination.

An important outgrowth of these studies was a technical development that allows for the simultaneous screening of up to four peptides. While the standard protocol for these experiments involves stimulating donor PBMC with a single peptide, the modified protocol utilizes stimulations with up to four different peptides (Appendix 2). Following multiple rounds of stimulation, the T cell cultures are tested against a pool of all the peptides, with those cultures exhibiting reactivity subsequently tested against the individual peptides. Using this approach we did not identify any additional HLA-A1 or HLA-A3-restricted peptides (Appendix 2), and for the clarity of presentation we have incorporated these results into section 2.4 below.

2.4. Repeat 2.3 for peptides associated with HLA-A2, B7, and B8. The order in which this is done will be dictated by order in which patient material becomes sufficiently available to conduct the experiments. (Months 13-36)

TAL0319, TAL0511, and TAL567 CTL were used to test HLA-A2 restricted peptides including four from FBP and three from mesothelin (Tables 6, 12, 14). The TAL0319 had high background reactivity on the unpulsed C1R-A2, but the this activity was not seen with the TAL0511 and TAL567 CTL. The results demonstrate that none of the peptides are reproducibly recognized by the CTL.

Screening HLA-A2, B7, and B8 restricted peptides also continued as described in 2.4 above.

Each peptide listed in Appendix 2, Table 4 was synthesized and tested for its ability to prime a peptide-specific CTL response using PBL obtained from three to nine healthy donors. PBL were stimulated with peptide-pulsed, autologous mDC, generally in 48 individual microcultures per donor. Following the fourth restimulation, the individual cultures were tested for reactivity with peptide-pulsed target cells in a ^{51}Cr-release assay. Cultures, with killing that was more than 20% above that found on the target cells not pulsed with peptide, were selected for additional characterization. Initially, individual microcultures were stimulated with only a single peptide, and these cultures identified two peptides for further study including the HLA-A2-restricted peptide SLGWLFLLL and the HLA-B8-restricted peptide LSRLSNRLL (Appendix 2, Table 4). Selected cultures reactive with SLGWLFLLL and LSRLSNRLL were expanded with anti-CD3 antibody for further analysis. The reactivities of six SLGWLFLLL cultures are shown in Appendix 2, Fig. 1. The culture reactive with the LSRLSNRLL peptide lost its peptide-specificity following expansion, and although it recognized tumor, it was not studied further.

Screening individual test peptides for their ability to stimulate a CTL response is an inherently time and resource intensive endeavor. To determine the feasibility of screening multiple peptides simultaneously, PBL of one of the donors reactive against the SLGWLFLLL peptide in association with HLA-A2 was also stimulated with a mix of four peptides including SLGWLFLLL. Because of limitations on the number of responder cells in each microculture, the initial screening was performed against targets pulsed with all four peptides, and this yielded positive cultures (Appendix 2, Table 4). Cultures with cytotoxic reactivity were then expanded with anti-CD3 and tested against targets individually pulsed with each of the peptides used in the stimulation. Not only was a response against SLGWLFLLL in the mix detected, but a response was also measured against LLLRLECNV in association with HLA-A2, thus validating that multiple peptides can be tested simultaneously for their ability to induce a CTL response (Appendix 2, Table 4; Fig. 2A, C). A similar peptide mix lacking the SLGWLFLLL peptide was also used to identify another LLLRLECNV reactive culture in an additional donor (Appendix 2, Table 4). Likewise, a mix of HLA-B7 peptides led to the identification of LPAQEGAPT as a candidate epitope (Appendix 2, Table 4; Appendix 2, Fig. 2B, D). Although LPAQEGAPT-reactive CTL were shown to recognize tumor (data not shown), their specificity could not be confirmed in cold target inhibition experiments and results with this peptide are not discussed further.

To confirm that the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2, the respective CTL cultures were tested against a panel of target cells that were either matched or unmatched for the class I MHC molecule of interest. Included among these targets were C1R-A2 and C1R-B7 which are class I MHC gene transfectants (HLA-A*0201 and HLA-B*0702, respectively) of the class I MHC null cell line, Hmy2.C1R. These transfectants can be used to unambiguously determine the class I MHC restriction of a CTL line or clone. The target cells were incubated in the presence of the test peptide and then tested for their susceptibility to lysis by the peptide-specific CTL.

CTL lines 69C4 and 22E5, specific for the SLGWLFLLL peptide, recognized the HLA-A2 positive cell lines T2 and C1R-A2 when pulsed with peptide, but did not recognize the HLA-A2 negative MST and C1R-B7 lines when pulsed with peptide (Appendix 2, Fig. 3A, 3B). The same results were obtained with the CTL line 82C8, specific for the LLLRLECNV peptide (Appendix 2, Fig. 3C). These results demonstrate that both the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2.

The relative affinity of the peptide-specific CTL lines was determined by testing the ability of the CTL to recognize target cells incubated with 10-fold dilutions of peptide, beginning at 10 µg/ml (~10 µM). CTL lines recognizing the SLGWLFLLL peptide showed a broad range of peptide concentrations over which half-maximal killing was achieved, with most having half-maximal activity between 0.1 and 10 nM (Appendix 2, Fig. 4A). CTL lines recognizing the LLLRLECNV peptide had half maximal activity between 1 and 20 nM (Appendix 2, Fig. 4B).

To determine if the peptide-specific CTL also recognize tumor cells, the CTL were tested for their ability to recognize tumors expressing both the appropriate class I MHC molecule and the TAG gene. CTL lines 22E5 and 69C4 (SLGWLFLLL specific) recognized some, but not all, tumors expressing both HLA-A2 and the TAG genes, however, tumors expressing only HLA-A2 or the tumor antigen alone were not recognized (Appendix 2, Fig. 5A, 5B, and data not shown). Likewise,–CTL line 82C8 (LLLRLECNV specific) recognized some, but not all tumor lines expressing HLA-A2 and the TAG genes, however, tumors expressing either the HLA-A2 or the tumor antigen alone were not recognized (Appendix 2, Fig. 5C and data not shown).

The tumor reactivity of the CTL lines was further confirmed in cold target inhibition experiments. The recognition of ^{51}Cr-lableled DM13 tumor cells (HLA-A2^{+}, TAG^{+}) by the CTL line 69C4 (HLA-A2-restricted, SLGWLFLLL-specific) was inhibited by unlabeled SLGWLFLLL-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Appendix 2, Fig. 6A). Similarly, the recognition of ^{51}Cr-lableled DM6 tumor cells (HLA-A2^{+}, TAG^{+}) by the CTL line 82C8 (HLA-A2-restricted, LLLRLECNV-specific) was inhibited by unlabeled LLLRLECNV-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Appendix 2, Fig. 6B). These results confirm that the TAG-derived SLGWLFLLL and LLLRLECNV peptides are naturally processed and presented by HLA-A2 on tumor cells.

2.5 Determine the ability of CTL generated in 1.2 above to recognize target cells transfected or infected with the gene of interest and identify the peptide antigen. Confirm with SRM mass spectrometry. (Months 6-36)

As indicated in 1.2 above, we did not obtain CTL that can be used for this aim and therefore, no results are available.

Table 1. Peptides Synthesized for Testing[a]

Source Protein	Tested Peptides Segregated by Class I MHC Binding Molecule				
	HLA-A1	HLA-A2	HLA-A3	HLA-B7	HLA-B8
Binding Motifs[b]	--E-----Y D	-M------V L L I	-M------Y L K	-PR-----L I	--K-K---- R R
FBP	**PNEE**VARFY	**LLLV**WVAVV **FLLS**LALML **LLSL**ALMLL **SLAL**MLLWL	**LLNV**SMNAK **RVLN**VPLSK **YLYR**FNWNH **AVVG**EAQTR	**GPWA**AWPFL **AQRM**TTQLL **WPFL**LSLAL	IAWARTELL IAWARTELL APASKRHFI QSWRKERVL
HER-2/neu (HER)	**HLDM**LRHLY **LLDI**DETEY **VSEF**SRMAR		**ILIK**RRQQK **ILKE**TELRK	**DVRL**VHRDL **LPAS**PETHL	ESRPRFREL LIKRRQQKI DLLEKGERL SPKANKEIL
Mesothelin (Mes)	**EIDE**SLIFY **TLDT**LTAFY	**FLLF**SLGWV **SLLF**LLFSL **VLPL**TVAEV	**ELAV**ALAQK **ALQG**GGPPY	**APTE**DLKAL **RVRE**LAVAL **GPGP**VLTVL **GPVL**TVLAL	ILRQRQDDL VLKHKLDEL
TAG	**ESE**RGLPAS **NLE**PLVSRD **SRD**PPASAS	**TLS**RLSNRL **LLLR**LECNV **SLG**WLFLLL **FLL**LLNSTT	GLPASTLSR LLLLNSTTK	**LPAQ**EGAPT **VQR**RAEGLL **LPAS**TLSRL **LSR**LSNRLL **DPP**ASASLF	TVQRRAEGL LSRLSNRLL VQRRAEGLL

[a]Bolded amino acids are the code used for each peptide in subsequent tables.

Table 2. TAL546 Specificity[a]

	Target Cells (Class I MHC)					
E:T	ES-2 HLA-A3	SKOV3.A2 HLA-A2, A3	TOV112D HLA-A3	SKOV3 HLA-A3	OV90 HLA-A2	TTB6 HLA-A2
60	0.4[b]	5.6	-7.8	2.7	2.1	1.6
30	0.0	1.6	-7.8	-1.2	0.4	2.1
15	0.7	1.3	-7.8	-0.5	-1.3	2.0
7.5	-0.3	-0.5	-7.8	-1.8	-0.7	0.7

[a]TAL546 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 3. TAL572 Specificity[a]

	Target Cells (Class I MHC)								
E:T	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR3 HLA-A2	SK-OV-3.A2 HLA-A2	TPF572 Autologous	TTB6 HLA-A2	TOV-112D HLA-A3	ES-2 HLA-A3
80	10.0	11.0	7.3	3.2	20.0	63.0	2.0	22.3	10.7
40	4.0	3.7	1.2	3.3	11.8	56.2	1.9	17.5	5.2

[a]TAL519 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 4. TAL11770 Specificity[a]

	Target Cells (Class I MHC)								
E:T	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR3 HLA-A2	SK-OV-3.A2 HLA-A2	TTB-6 HLA-A2	OAT 11770 HLA-A2	TOV-112D HLA-A3	ES-2 HLA-A3
80	9.1[b]	11.3	12.1	6.6	11.7	9.1	10.2	44.6	12.7
40	4.7	9.9	3.4	2.6	10.6	0.7	8.2	35.5	5.1
20	5.7	6.7	2.2	2.0	6.4	0.4	9.4	9.3	4.9
10	3.3	3.5	-0.4	2.4	3.8	-0.2	5.8	6.0	0.1

[a]TAL11770 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 5. TAL0319 Specificity[a]

	Target Cells (Class I MHC)							
E:T	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR-3 HLA-A2	SKOV3.A2 HLA-A2	TTB6 HLA-A2	OAT0319 HLA-A2	SKOV3 HLA-A3
80	66.0[b]	89.4	58.1	60.6	72.6	62.9	8.8	49.7
20	53.6	66.2	42.9	48.0	63.7	45.3	3.7	26.5

[a]TAL0319 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 6. TAL0319 Reactivity Against HLA-A2-Restricted Peptides[a]

	Target Cells											
Peptide: Source: Status: E:T	C1R-A2 + LLLV FBP	C1R-A2 + FLLS FBP	C1R-A2 + LLSL FBP	C1R-A2 + SLAL FBP	C1R-A2 + FLLF Mes	C1R-A2 + SLLF Mes	C1R-A2 + VLPL Mes	C1R-A2 + SLG TAG Known	C1R-A2 + KIF HER Known	C1R-A2 + EIW FBP Known	C1R-A2	K562
40	78.6 [b]	77.7	82.7	73.9	78.5	77.0	83.1	82.6	84.6	86.3	89.0	66.7
10	67.7	74.2	64.7	64.7	70.9	68.8	72.4	77.7	58.5	64.5	60.8	62.0

[a]TAL0319 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 7. TAL0406 Specificity[a]

	Target Cells (Class I MHC)					
E:T	ES-2 HLA-A3	SKOV3 HLA-A3	SW626 HLA-A3	TOV-112D HLA-A3	OAT0406 HLA-A3	OV-90 HLA-A2
80	87.7 [b]	42.9	75.0	88.2	19.8	51.1
20	63.0	29.3	58.4	75.6	6.4	38.7

[a]TAL0406 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 8. TAL0406 Reactivity Against HLA-A1-Restricted Peptides[a]

	Target Cells								
Peptide: Source: Status: E:T	C1R-A1 + PNEE FBP	C1R-A1 + HLDM HER	C1R-A1 + LLDI HER	C1R-A1 + VSEF HER	C1R-A1 + EIDE Mes	C1R-A1 + TLDT Mes	C1R-A1 + EADP MAGE-1 Known	C1R-A1	K562
40	54.3 [b]	50.7	59.4	53.1	61.8	57.9	57.4	55.6	78.2
10	30.7	33.9	33.5	30.9	25.1	29.1	32.5	32.2	53.3

[a]TAL567 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 9. TAL0406 Reactivity Against HLA-A3-Restricted Peptides[a]

	Target Cells											
Peptide: Source: Status: E:T	C1R-A3 + LLNV FBP	C1R-A3 + RVLN FBP	C1R-A3 + YLYR FBP	C1R-A3 + AVVG FBP	C1R-A3 + ILIK HER	C1R-A3 + ILKE HER	C1R-A3 + ELAV Mes	C1R-A3 + ALQG Mes	C1R-A3 + SLF MAGE-A1 Known	C1R-A3 + VLR HER Known	C1R-A3	K562
40	43.0[b]	49.2	45.1	51.6	53.8	46.8	59.2	43.5	43.0	49.7	51.3	78.2
10	28.3	27.3	23.1	23.1	26.3	28.2	36.2	17.4	22.6	27.9	23.1	53.3

[a]TAL0406 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 10. TAL0511 Specificity[a]

	Target Cells (Class I MHC)												
E:T	CAOV-4 HLA-A2	COV413 HLA-A2	ES-2 HLA-A3	OV-90 HLA-A2	OVCAR-3 HLA-A2	SK-OV-3 HLA-A3	SK-OV-3.A2 HLA-A2, A3	SW626 HLA-A3	TOV-112D HLA-A3	TTB6 HLA-A2	OAT0319 HLA-A2	OAT0511 HLA-A2, A3	TOV-21G $A2^-$, $A3^-$
40	3.9[b]	10.2	5.0	3.4	0.8	2.9	3.1	1.4	6.9	4.0	13.7	18.1	32.2
20	2.0	5.3	2.7	3.2	1.7	2.0	1.6	-0.2	4.8	3.0	6.5	13.1	22.9
10	1.0	3.5	0.8	3.2	1.1	-0.3	0.2	-2.4	3.1	2.2	1.1	7.8	11.1
5	0.0	1.1	-0.3	0.6	-0.5	-0.7	-1.4	-2.1	-1.5	1.1	-0.1	2.5	1.7

[a]TAL0511 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 11. TAL0511 Reactivity Against HLA-A3-Restricted Peptides[a]

	Target Cells											
Peptide: Source: Status: E:T	C1R-A3 + LLNV FBP	C1R-A3 + RVLN FBP	C1R-A3 + YLYR FBP	C1R-A3 + AVVG FBP	C1R-A3 + ILIK HER	C1R-A3 + ILKE HER	C1R-A3 + ELAV Mes	C1R-A3 + ALQG Mes	C1R-A3 + SLF MAGE-A1 Known	C1R-A3 + VLR HER Known	C1R-A3	K562
40	14.4[b]	15.3	16.6	18.0	16.6	15.3	17.8	16.3	12.4	14.7	13.0	12.6
20	12.9	10.0	12.2	10.2	9.0	10.5	10.8	9.4	8.4	9.5	9.4	9.2
10	6.0	6.4	8.3	7.4	6.4	6.7	8.6	6.7	6.1	6.0	5.6	6.4
5	3.6	4.8	3.8	6.5	4.5	4.3	4.6	3.4	0.9	1.4	3.1	2.7

[a]TAL0511 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 12. TAL0511 Reactivity Against HLA-A2-Restricted Peptides[a]

	Target Cells											
Peptide: Source: Status: E:T	C1R-A2 + LLLV FBP	C1R-A2 + FLLS FBP	C1R-A2 + LLSL FBP	C1R-A2 + SLAL FBP	C1R-A2 + FLLF Mes	C1R-A2 + SLLF Mes	C1R-A2 + VLPL Mes	C1R-A2 + SLG TAG Known	C1R-A2 + KIF HER Known	C1R-A2 + EIW FBP Known	C1R-A2	K562
40	25.0[b]	33.9	31.5	38.1	27.5	29.6	32.8	32.2	31.1	31.1	31.2	12.6
20	20.0	21.8	22.2	23.5	19.9	18.6	24.1	23.4	24.3	23.2	21.5	9.2
10	16.3	16.7	16.9	17.6	16.4	15.1	15.6	17.3	17.6	16.1	14.2	6.4
5	12.1	10.1	14.3	11.2	7.5	10.1	12.1	11.2	14.3	10.9	8.1	2.7

[a]TAL0511 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 13. TAL567 Specificity[a]

	Target Cells (Class I MHC)								
E:T	CAOV4 HLA-A2	COV413 HLA-A2	OV90 HLA-A2	OVCAR3 HLA-A2	SKOV3.A2 HLA-A2,3	TTB6 HLA-A2	TPF567 HLA-A2	TPF568 HLA-A2	SKOV3 HLA-A3
80	5.5[b]	0.9	3.5	2.5	10.5	1.5	53.1	54.4	0.8
40	3.9	0.7	1.0	1.0	8.6	0.9	45.5	43.0	0.6
20	2.1	-0.5	1.6	1.4	5.6	0.2	33.0	29.7	-0.2
10	0.3	0.1	1.0	-0.3	1.0	1.8	20.1	17.5	-0.3

[a]TAL567 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 14. TAL567 Reactivity Against HLA-A2-Restricted Peptides[a]

	Target Cells					
Peptide: Source: Status: E:T	C1R-A2 + FLLF Mes	C1R-A2 + SLLF Mes	C1R-A2 + VLPL Mes	C1R-A2 + KIF HER Known	C1R-A2	K562
40	3.7[b]	4.1	6.0	6.3	4.7	6.9
20	3.6	2.5	3.3	4.3	6.5	4.5
10	1.8	-1.3	2.1	2.3	4.8	2.3
5	-0.7	-1.4	1.2	-0.3	2.5	1.2

[a]TAL567 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 15. TAL519 Specificity[a]

	Target Cells (Class I MHC)								
E:T	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR3 HLA-A2	SK-OV-3.A2 HLA-A2	SW626 HLA-A2	TPF519 Autologous	TTB6 HLA-A2	ES-2 HLA-A3
80	8.8[b]	3.8	8.1	5.3	15.0	0.7	7.9	7.0	17.5
40	3.7	1.5	5.5	2.5	11.4	0.6	5.3	3.6	13.9

[a]TAL519 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

Table 16. VMM528 Recognition of DAC Treated Cells[a]

E:T	Target Cells (Class I MHC)							
	COV413 (HLA-A2$^+$)		OVCAR3 (HLA-A2$^+$)		TOV-112D (HLA-A2$^-$)		SW626 (HLA-A2$^-$)	
	-DAC	+DAC	-DAC	+DAC	-DAC	+DAC	-DAC	+DAC
20	4.8[b]	27.2	14.0	40.2	8.5	12.2	3.7	0.5
10	3.9	24.4	9.3	38.8	7.4	8.3	1.1	-0.6
5	2.2	17.9	5.8	33.7	2.0	0.4	1.9	1.1
2.5	1.8	9.3	5.0	30.6	2.8	1.6	-1.0	-1.2

[a]VMM528 CTL were tested in a ^{51}Cr-release assay against the indicated targets.
[b]% Specific ^{51}Cr-release.

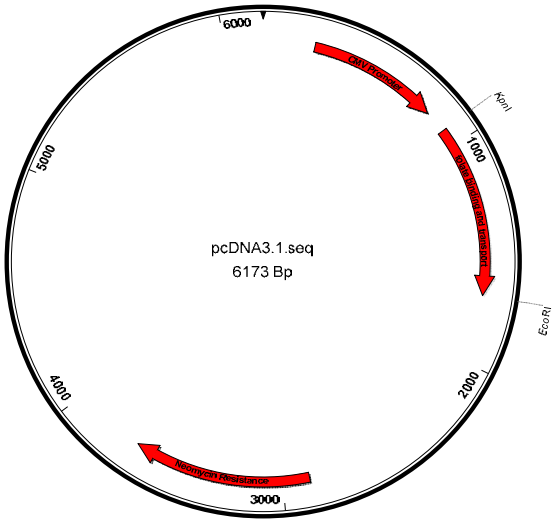

Fig. 1. FBP gene construct in pcDNA3.1. The FBP gene was cloned as a *Kpn*I/*Eco*RI fragment.

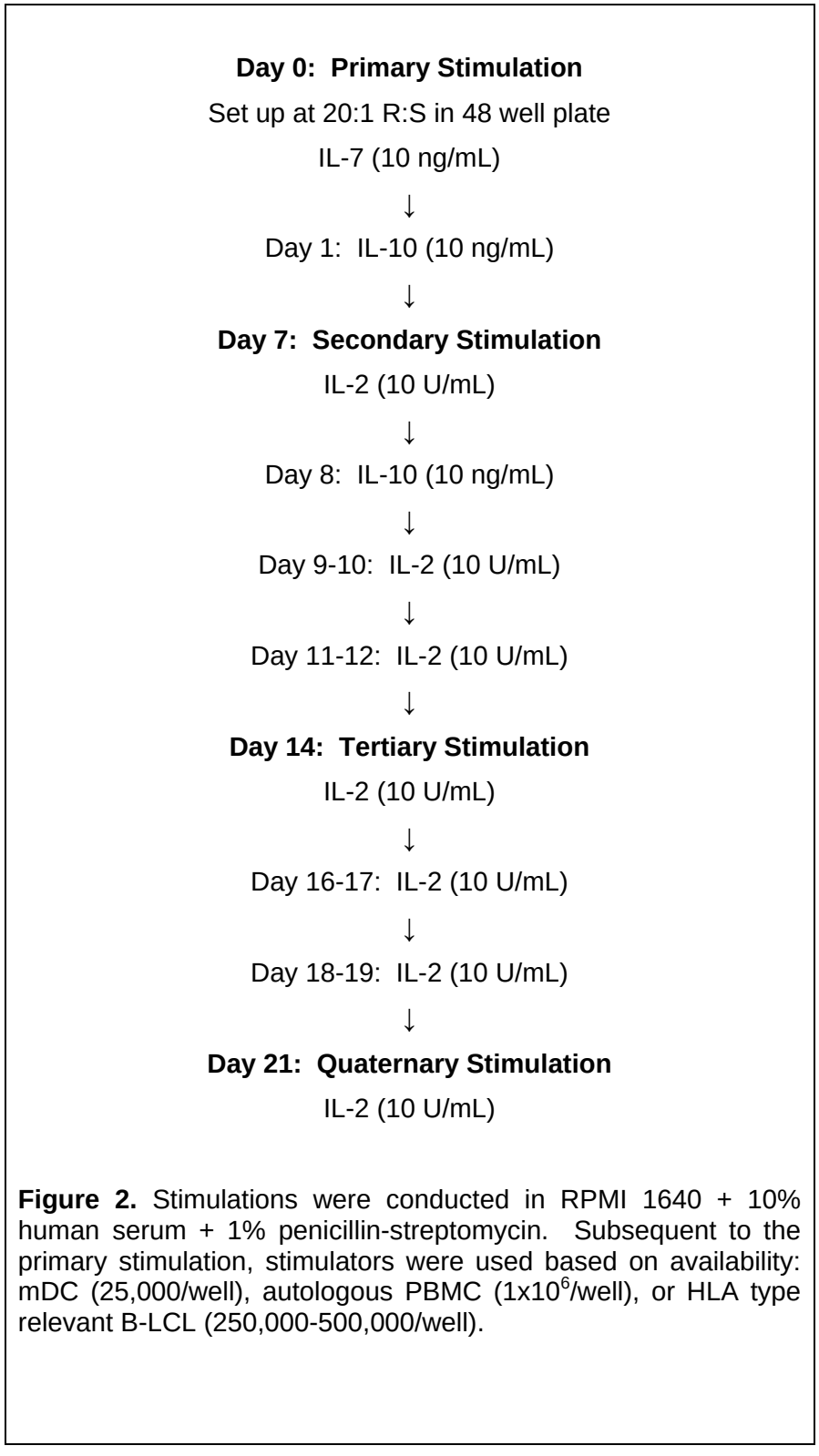

Figure 2. Stimulations were conducted in RPMI 1640 + 10% human serum + 1% penicillin-streptomycin. Subsequent to the primary stimulation, stimulators were used based on availability: mDC (25,000/well), autologous PBMC ($1x10^6$/well), or HLA type relevant B-LCL (250,000-500,000/well).

^{51}Chromium-release assay post 4º stimulation

Each well is tested in duplicate with 2000 target cells per well at 40:1 and 10:1 E:T. Target cells are labeled for 2 hours, washed and peptide pulsed for 1 hour, and co-cultured with effector cells for 4 hours, after which supernatant is harvested. CPM measured via gamma counter.

↓

Positive well selection

A well is generally considered positive when percent specific release is 20% points over background.

↓

5º stimulation

↓

^{51}Chromium-release assay

An assay using a two-fold geometric series dilution in triplicate is conducted as described above.

↓

T cell expansion with anti-CD3 mAb

A T25 flask is seeded with 100,000 cells from positive well + 25 x 10^6 PBMC in RPMI 1640 + 10% FBS + 2 mM L-glutamine + 1% penicillin-streptomycin + 10 ng/mL anti-CD3 mAb + 25 U/mL IL-2.

↓

Further investigation of peptide specificity

Peptide titration, tumor assay, cold target inhibition assay, etc.

Figure 3. Evaluation of peptide specificity procedures.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized eleven ovarian cancer cell lines:
  - Established the class I MHC genotype, class I MHC phenotype (overall class I MHC expression, HLA-A2, HLA-A3, and HLA-B7), and class II MHC phenotype (overall expression) of eleven ovarian cancer cell lines
  - Characterized the eleven ovarian cancer cell lines for the expression of the FBP, Her-2/neu, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, TAG-2c, and CEA antigens
  - Demonstrated that there is not always a linear relationship between mRNA and protein expression for Her-2/neu, FBP, and MAGE-A1 – in the latter case this may be do to cross reactivity of the anti-MAGE-A1 antibody on other MAGE family members
  - Demonstrated that TGF-β1 and TGF-β2 are variously produced by the ovarian cancer lines, but that TGF-β3 is only minimally produced
  - Demonstrated that IL-10 was only produced by the ovarian cancer cell line SW626
  - Demonstrated that the pattern of expression of cytokeratins differs among ovarian cancer cell lines and that no single pattern uniquely identifies ovarian cancer cells
  - Demonstrated that CD90, a marker associated with fibroblasts, is sometimes expressed on ovarian cancer cells
  - Complete characterization of the class I MHC and tumor antigen expression of 29 ovarian cancer patient archival samples – these samples will be used for establishing additional ovarian cancer cell lines and for establishing ovarian cancer cell-specific CTL
- CTL characterization
  - Established a streamlined and efficient methodology for assessing the immunogenicity of peptides predicted to be presented in association with defined class I MHC molecules such that up to four peptides can be simultaneously tested
  - Established CTL lines from nine ovarian cancer patients, however, none of these appeared to recognize shared antigens and thus were not useful antigen identification – this “negative” result is important because it suggest that either there are only a limited number of shared antigens expressed by ovarian cancer cells or that the immunosuppressive environment of ovarian cancer prevents the development of T cells that recognize such antigens
- Antigen identification
  - Two HLA-A2-restricted epitopes (SLGWLFLLL and LLLRLECNV) derived from the cancer/testis antigen TAG-1 have been identified as ovarian cancer antigens
  - One HLA-B7-restricted epitope (LPAQEGAPT) derived from TAG-1 was tentatively identified as an ovarian cancer antigen, but additionally confirmatory work is needed before this can be stated with confidence
  - Demonstrated that none of the generated CTL recognize any of the predicted peptide antigens from FBP, Her-2/neu, or mesothelin
  - Demonstrated that treatment of ovarian cancer cell lines with DAC is capable of up-regulating the expression of cancer/testis antigens and class I MHC molecule expression by the cell lines – this is significant because ovarian cancer cells that would otherwise

escape detection by T cells can now be targeted using the large number of antigenic peptides that have already been defined

- Cloned the FBP gene

REPORTABLE OUTCOMES

Publications

Carr, T.M., Adair, S.J., Fink, M.J., and Hogan, K.T. (2008) Immunological profiling of a panel of ovarian cancer cell lines. Cancer Immunol. Immunother. 57:31-42.

Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2007) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 -restricted epitopes. J. Immunother. 31:7-17.

Adair, S.J., and Hogan K.T. (2008) Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. Cancer Immunol. Immunother. In press. DOI: 10.1007/s00262-008-0582-6.

Abstracts

Adair, S.J., Carr, T.M., and Hogan, K.T. (2005) Identification of cytotoxic T lymphocyte epitopes derived from the cancer/testis antigen, TAG. iSBTc Annual Meeting. J. Immunotherapy 28:639.

Hogan, K.T., Carr, T.M., Adair, S.J., and Fink, M.J. (2007) Immunological characterization of eleven ovarian cancer cell lines. iSBTc Annual Meeting. J. Immunotherapy 30:888.

Funding Applied For

NIH/NCI, 1R21CA140744-01, Ovarian Cancer Stem Cells as Targets for Immunotherapy

Marsha Rivkin Center for Ovarian Cancer Research, Antigenic Targets on Ovarian Cancer Stem Cells

CONCLUSION

The ability to identify ovarian cancer antigens for the development of a therapeutic vaccine is critically dependent upon the availability of well-characterized ovarian cancer cell lines, which until now, have not been available. These lines are used both to stimulate CTL and to act as targets when testing the specificity of existing CTL. The characterization of the eleven ovarian cancer cell lines in this project fills the previously existing void and provides us and other ovarian cancer researchers with a needed tool that will facilitate ongoing efforts to identify additional ovarian cancer antigens.

The inability to generate CTL that recognize a shared antigen or any of the predicted antigens is problematic from a research perspective in which the desire is to identify additional antigens for inclusion in a therapeutic vaccine for the treatment of ovarian cancer. This may in fact reflect a paucity of such antigens. From the perspective of vaccine development this is an important finding as it would indicate that development of a vaccine is not technically feasible because of the lack of common antigens.

Conversely, we identified two HLA-A2-restricted antigens that are shared by ovarian cancer cells. We have also found that high levels of expression of diverse cancer/testis antigens can be induced by treating ovarian cancer cells with DAC. These two findings taken together are significant because they indicate that ovarian cancer cells are in fact capable of expressing shared tumor antigens. This in turn suggest that future research should be directed towards defining the mechanisms that block a robust T cell response to these antigens. From a clinical perspective, these results indicate that combination therapy involving a cancer vaccine and a DNA demethylating agent may have synergistic effects when the vaccine targets antigens that can be upregulated by a DNA demethylating agent.

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BIBLIOGRAPHY OF PUBLICATIONS/ABSTRACTS

Publications

1. Carr, T.M., Adair, S.J., Fink, M.J., and Hogan, K.T. (2008) Immunological profiling of a panel of ovarian cancer cell lines. Cancer Immunol. Immunother. 57:31-42.
2. Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2008) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 -restricted epitopes. J. Immunother. 31:7-17.
3. Adair, S.J., and Hogan K.T. (2008) Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. Cancer Immunol. Immunother. Published on-line DOI:10.1007/s00262-008-0582-6. (Includes 3 pages of supplemental data)

Abstracts

1. Adair, S.J., Carr, T.M., and Hogan, K.T. (2005) Identification of cytotoxic T lymphocyte epitopes derived from the cancer/testis antigen, TAG. iSBTc Annual Meeting. J. Immunother. 28:639.
2. Hogan, K.T., Carr, T.M., Adair, S.J., and Fink, M.J. (2007) Immunological characterization of eleven ovarian cancer cell lines. iSBTc Annual Meeting. J. Immunother. 30:888.

LIST OF PERSONNEL RECEIVING PAY FROM RESEARCH EFFORT

Kevin T. Hogan, Ph.D.

William P. Irvin, M.D.

James R. McGlothlin

Sara J. Adair

Tiffany M. Carr

APPENDIX

Appendix 1: Carr, T.M., Adair, S.J., Fink, M.J., and Hogan, K.T. (2008) Immunological profiling of a panel of ovarian cancer cell lines. Cancer Immunol. Immunother. 57:31-42.

Appendix 2: Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2008) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 -restricted epitopes. J. Immunother. 31:7-17.

Appendix 3: Adair, S.J., and Hogan K.T. (2008) Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. Cancer Immunol. Immunother. Published on-line DOI:10.1007/s00262-008-0582-6. (Includes 3 pages of supplemental data)

Appendix 4: Adair, S.J., Carr, T.M., and Hogan, K.T. (2005) Identification of cytotoxic T lymphocyte epitopes derived from the cancer/testis antigen, TAG. iSBTc Annual Meeting. J. Immunother. 28:639.

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Cancer Immunol Immunother (2008) 57:31–42
DOI 10.1007/s00262-007-0347-7

APPENDIX 1

ORIGINAL ARTICLE

Immunological profiling of a panel of human ovarian cancer cell lines

Tiffany M. Carr · Sara J. Adair · Mitsú J. Fink · Kevin T. Hogan

Received: 12 April 2007 / Accepted: 23 May 2007 / Published online: 20 June 2007

Abstract
Purpose The efficient identification of peptide antigens recognized by ovarian cancer-specific cytotoxic T lymphocytes (CTL) requires the use of well-characterized ovarian cancer cell lines. To develop such a panel of cell lines, 11 ovarian cancer cell lines were characterized for the expression of class I and class II major histocompatibility complex (MHC)-encoded molecules, 15 tumor antigens, and immunosuppressive cytokines [transforming growth factor β (TGF-β) and IL-10].
Methods Class I MHC gene expression was determined by polymerase chain reaction (PCR), and class I and class II MHC protein expression was determined by flow cytometry. Tumor antigen expression was determined by a combination of polymerase chain reaction (PCR) and flow cytometry. Cytokine expression was determined by ELISA.
Results Each of the ovarian cancer cell lines expresses cytokeratins, although each cell line does not express the same cytokeratins. One of the lines expresses CD90, which is associated with a fibroblast lineage. Each of the cell lines expresses low to moderate amounts of class I MHC molecules, and several of them express low to moderate amounts of class II MHC molecules. Using a combination of PCR and flow cytometry, it was determined that each cell line expressed between six and thirteen of fifteen antigens tested. Little to no TGF-β3 was produced by any of the cell lines, TGF-β1 was produced by three of the cell lines, TGF-β2 was produced by all of the cell lines, with four of the cell lines producing large amounts of the latent form of the molecule, and IL-10 was produced by one of the cell lines.
Conclusions Each of the 11 ovarian cancer lines is characterized by a unique expression pattern of epithelial/fibroblast markers, MHC molecules, tumor antigens, and immunosuppressive cytokines. Knowledge of these unique expression patterns will increase the usefulness of these cell lines in identifying the antigens recognized by ovarian cancer-specific CTL.

Keywords Ovarian cancer · Class I MHC molecules · Cancer antigens · Immune suppression · Cytokine

Abbreviations

B-LCL	B-lymphoblastoid cell line
CEA	Carcinoembryonic antigen
CTL	Cytotoxic T lymphocyte
Ck	Cytokeratin
FBP	Folate binding protein
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
TGF-β	Transforming growth factor β

Introduction

Early studies indicated that T cells infiltrate solid, ovarian tumors [24, 27]. Immunohistochemical analysis subsequently showed $CD8^+$ T cell infiltrates in biopsy samples [38], and flow cytometry showed that $CD3^+$ T cells are the major leukocyte population detected in both tumor-infiltrating lymphocytes and tumor-associated lymphocytes [56].

T. M. Carr · S. J. Adair · M. J. Fink · K. T. Hogan (✉)
Department of Surgery and the Human Immune Therapy Center, University of Virginia,
Box 801359, Charlottesville, VA 22908, USA
e-mail: kh6s@virginia.edu

An immunohistochemical analysis of advanced-stage ovarian carcinoma specimens indicated that the presence of tumor-infiltrating $CD3^+$ T lymphocytes strongly correlates with increased time of survival and increased time to recurrence of stage III and IV patients following surgical resection and chemotherapy [66]. More recently, in ovarian cancer tumors and ascites, it was shown that the presence of $CD4^+25^+FOXP3^+$ T regulatory cells is correlated with poor survival [11]. Taken together, this evidence suggests that T cell-mediated immunity plays a significant role in the pathology of ovarian cancer, and further suggests that T cell-mediated immunotherapy might be a viable approach to treating the disease.

One form of T cell-mediated immunotherapy that is currently under development involves the immunization of ovarian cancer patients with antigenic peptides that bind to class I MHC molecules and stimulate a tumor reactive CTL response [26, 43, 47]. While a large number of peptide antigens are available for clinical trials in melanoma, comparatively few peptides are available for clinical trials in ovarian cancer [39]. Tumor antigens known to be expressed in ovarian cancer and for which antigenic peptides have been identified include Her-2/neu [14, 16, 46], folate binding protein (FBP) [29, 44, 45], the aminoenhancer of split protein [4], and the cancer/testis antigens NY-ESO-1 [40], LAGE-1 [40], MAGE-A1 [20, 65], and TAG [25].

Although a variety of approaches have been taken to identify peptide antigens that can be used to stimulate tumor reactive CTL [51, 58], all of these approaches ultimately rely on the availability of cell lines that have been characterized with respect to their expression of MHC molecules and tumor antigen proteins. As part of our effort to identify such antigens we have characterized eleven established ovarian cancer cell lines for their expression of class I and class II MHC molecules, and for their expression of the tumor antigens carcinoembryonic antigen (CEA), FBP, Her-2/neu, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, and TAG-2c. As these cell lines may also be used in attempts to stimulate a CTL response, we also determined if they express the immunosuppressive cytokines TGF-β1, TGF-β2, TGF-β3, and IL-10.

Materials and methods

Cell culture medium

RPMI-1640 was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (RPMI-10FBS).

Cell lines

The ovarian cancer lines CAOV-3 [10], CAOV-4 [28], ES-2 [32], OV-90 [48], OVCAR-3 [23], SK-OV-3 [17], SW626 [18], TOV-21G [48], and TOV-112D [48] were obtained from the ATCC (Manassas, VA). The ovarian cancer cell line TTB-6 was established at the University of Virginia, and the ovarian cancer cell line COV413 [30] was obtained from Dr. Angela Zarling (University of Virginia). The lines were maintained in RPMI-10FBS.

The B-lymphoblastoid cell line (B-LCL) JY (HLA-A2; −B7) was maintained in RPMI-10FBS. Class I MHC transfectants of Hmy2.C1R were maintained in RPMI-10FBS supplemented with 300 μg/ml G418 or 300 μg/ml hygromycin and included C1R-A1 (HLA-A1, hygromycin), C1R-A2 (HLA-A2, G418), and C1R-A3 (HLA-A3, G418). The skin fibroblast cell line CCD39SK was maintained in RPMI-10FBS containing 1 mM sodium pyruvate and 0.1 mM nonessential amino acids.

Primary antibodies

Monoclonal antibodies (mAb) BB7.2 (anti-HLA-A2, A69; γ_{2b}) [42], CR11-351 (anti-HLA-A2, A68, A69; γ_1) [52], GAP-A3 (anti-HLA-A3; γ_{2a}) [6], ME1-1.2 (anti-HLA-B7,B27; γ_1) [15], and W6/32 (anti-HLA-A, B, C; γ_{2a}) [41] were produced in our laboratory from the corresponding hybridoma and were used at a concentration of 10 μg/ml.

mAbs clone 3F257 (anti-MAGE-A1; γ_{2a}) (United States Biological, Swampscott, MA), Mov18/ZEL (anti-FBP; γ_1) (Axxora, San Diego, CA), TA-1 (anti-Her-2/neu; γ_1) (Calbiochem, La Jolla, CA), and COL-1 (anti-CEA; γ_{2a}) (BD Biosciences, San Diego, CA), were used at a concentration of 10 μg/ml. mAb AS02 (anti-CD90; γ_1) (Calbiochem) was used at a concentration of 5 μg/ml. mAb NCL-5D3 (anti-Ck 8/18; γ_{2a}) (MP Biomedicals, Solon, OH) was used at a 1:10 dilution.

Flow cytometry

For all of the antibody binding experiments except those using mAbs 3F257 and NCL-5D3, the primary antibodies were added to 2–5 × 10^5 cells and incubated for 30 min on ice. The cells were washed twice, 50 μl of a 1:50 dilution of sheep anti-mouse IgG-FITC (ICN, Irvine, CA) was then added, and the cells were incubated an additional 30 min on ice. The cells were then washed once, fixed with 2% paraformaldehyde in PBS, and analyzed on a FACSCalibur instrument (BD Biosciences, San Jose, CA).

Determination of 3F257 and NCL-5D3 antibody binding was done on cells that had been fixed and permeabilized according to the Cytofix/Cytoperm kit instructions (BD

Biosciences). Both the primary and secondary antibodies were diluted in perm/wash buffer.

TGF-β1, TGF-β2, TGF-β3, and IL-10 ELISA

Confluent cells were trypsinized, washed, and seeded at 3×10^6 cells per T75 flask in 8 ml of RPMI-10FBS. Forty-eight hours later, the media was collected, centrifuged, aliquoted, and stored at −80°C.

TGF-β1, TGF-β2, TGF-β3, and IL-10 concentrations were measured using DuoSet ELISA Development kits for each cytokine (R&D Systems, Minneapolis MN). Standard curves for each cytokine ranged from 2,000 to 15.625 pg/ml. Supernatants from each cell line were assayed in duplicate according to the manufacturer's instructions. Concentrations of active and active plus latent TGF-β were measured for all three isoforms. The TGF-β isoforms were activated in accordance with the protocol provided in the TGF-β1 kit. Following the addition of stop solution, absorbances were measured (450 nm test wavelength, 540 nm reference wavelength) on a DYNEX Technologies MRX II microplate reader (Chantilly, VA).

DNA, RNA, and cDNA preparation

DNA was obtained from 5×10^6 cells using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was prepared from 2–10×10^6 cells using the RNeasy Mini kit (Qiagen) as per the kit instructions. DNA and RNA were quantified by absorbance at 260 nm. Total RNA was converted to cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA).

Class I MHC gene typing

Class I MHC typing was done using the Micro SSP Generic HLA Class I typing tray SSP1L (One Lambda, Inc., Canoga Park, CA).

Polymerase chain reaction

Primer sequences used are shown in Table 1 and are as previously published for GAPDH [33], MAGE-A1 [8], MAGE-A2 [13], MAGE-A3 [12], MAGE-A4 [12], MAGE-A6 [12], MAGE-A10 [12], MAGE-A12 [12], NY-ESO-1 [64], TAG-1 [25], TAG-2a [25], TAG-2b [25], and TAG-2c [25]. Primers for FBP and Her-2/neu were designed using PrimerSelect software (DNASTAR, Inc., Madison, WI).

PCR was performed on 250 ng of cDNA using Platinum *Taq* High Fidelity (Invitrogen). The PCR mixes were heated to 94°C for 2 min, 30 and 40 cycles of amplification were performed (1 cycle = 30 s denaturation at 94°C, 30 s annealing at the temperature given in Table 1, 60 s extension at 72°C), and a final extension completed at 72°C for 5 min. The PCR products were visualized on ethidium bromide-stained agarose gels.

Human subjects research approval

This research was approved by the University of Virginia Human Investigation Committee in accordance with an

Table 1 Tumor antigen primer pairs used for PCR

Gene	Annealing temp (°C)	Product size (bp)	Forward primer (5′→3′)	Reverse primer (5′→3′)
GAPDH	60	598	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC
FBP	60	633	AGC CAG GCC CCG AGG ACA AGT	TGA GCA GCC ACA GCA GCA TTA GG
Her-2/neu	60	907	GCA CGG GCC CCA AGC ACT CTG ACT	ACT CGG CAT TCC TCC ACG CAC TCC
MAGE-A1	65	421	CGG CCG AAG GAA CCT GAC CCA G	GCT GGA ACC CTC ACT GGG TTG CC
MAGE-A2	68	317	AAG TAG GAC CCG AGG CAC TG	GAA GAG GAA GAA GCG GTC TG
MAGE-A3	66	725	TGG AGG ACC AGA GGC CCC C	GGA CGA TTA TCA GGA GGC CTG
MAGE-A4	68	446	GAG CAG ACA GGC CAA CCG	AAG GAC TCT GCG TCA GGC
MAGE-A6	69	727	TGG AGG ACC AGA GGC CCC C	CAG GAT GAT TAT CAG GAA GCC TGT
MAGE-A10	65	485	CAC AGA GCA GCA CTG AAG GAG	CTG GGT AAA GAC TCA CTG TCT GG
MAGE-A12	56	392	GGT GGA AGT GGT CCG CAT CG	GCC CTC CAC TGA TCT TTA GCA A
NY-ESO-1	66	458	GCG GCT TCA GGG CTG AAT GGA TG	AAG CCG TCC TCC TCC AGC GAC A
TAG-1	62	672	AGG AAG GGG CTC CCA CAG TGC	CCC AGG TTA GAA CGG TCA GCA GAA
TAG-2a	62	528	AGC GGC GGG CTG AAG GA	GAG GGT AGG GTG GTC ATT GTG TCA
TAG-2b	62	401	AGC GGC GGG CTG AAG GAC TC	CAG CAC AAC AGG AAC ATT CAG TGG
TAB-2c	62	536	AGC GGC GGG CTG AAG GA	GGG GGA TTT TAT TGC GGT GAA AGT

Primer references and cycling conditions are given in the "Materials and methods"

assurance filed with and approved by the Department of Health and Human Services.

Results

Ovarian cancer lines

Ten previously established ovarian cancer lines including CAOV-3 [10], CAOV-4 [28], COV413 [30], ES-2 [32], OV-90 [48], OVCAR-3 [23], SK-OV-3 [17], SW626 [18], TOV-21G [48], and TOV-112D [48] and one newly established ovarian cancer line (TTB-6) were used in this study. To confirm that TTB-6 was epithelial in origin, each of the lines was tested in flow cytometry for reactivity with mAb NCL-5D3 (anti-Ck 8/18) as an epithelial marker and mAb AS02 (anti-CD90) as a fibroblast marker. OVCAR-3, a well-studied ovarian cancer line was positive for Ck 8/18 expression and negative for CD90 expression, while CCD39SK, a skin fibroblast line obtained from the ATCC, demonstrated the opposite pattern of expression (Fig. 1). TTB-6 was positive for Ck 8/18 expression and negative for CD90 expression (Fig. 1), thus confirming the epithelial origin of the cell line. With the exception of ES-2 and TOV-112D, the remaining ovarian cancer lines were also Ck 8/18 positive and CD90 negative (data not shown). ES-2 did not express Ck 8/18, and greater than 90% of the cells were negative for CD90 (Fig. 1). TOV-112D did not express Ck 8/18, but did express CD90 (the small, CD90 negative population was present in two of five experiments).

Class I and II MHC expression

The class I MHC genotype of each of the ovarian cancer lines was determined by PCR analysis (Table 2). Because tumor cells frequently lose the expression of MHC molecules through a variety of mechanisms [57], we also sought to determine if class I MHC molecules could be detected on the surface of the cell lines. mAb W6/32, specific for an epitope present on all class I MHC molecules was used for the analysis (Fig. 2). Each of the lines was positive for class I MHC expression, albeit at levels that are low to moderate in comparison to the B-LCL, JY, which expresses high levels of class I MHC molecules. In the same analysis we also sought to determine if ovarian cancer cells express class II MHC molecules as determined by their ability to bind the class II MHC-specific mAb, L243 (Fig. 2). Most lines do not express class II MHC molecules, although low expression was detected on CAOV-3, CAOV-4, OVCAR-3, SW626, and a subpopulation of ES-2.

mAbs specific for some of the more prevalent class I MHC molecules in the population are available and were used to assess the expression of individual class I MHC molecules on the ovarian cancer lines (Table 3). Based on the genotype of the cells, each of the HLA-A2, -A3, -A68, -A69, and -B7 molecules were generally expressed at low to moderate levels in comparison to expression on C1R-A2, C1R-A3, C1R-B7, and JY. Expression of HLA-A2 and/or HLA-B7 on OV-90 and OVCAR-3 was particularly low, while expression of HLA-A3 and HLA-B7 on SW626 was quite high.

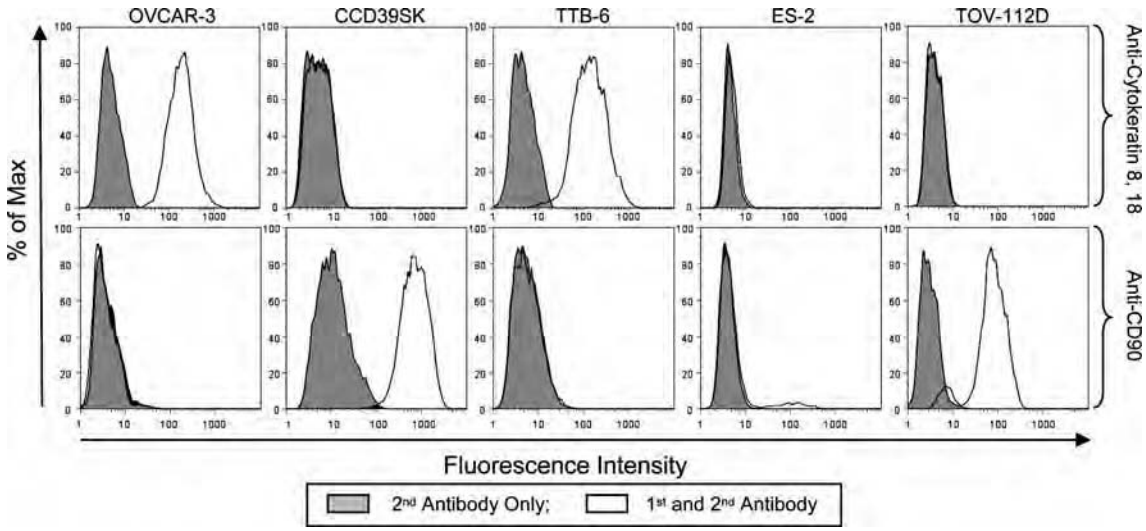

Fig. 1 Expression of epithelial and fibroblast markers by ovarian cancer lines. mAb NCL-5D3 (anti-cytokeratin 8, 18) was used to identify epithelial cells and AS02 (anti-CD90) was used to identify fibroblasts by flow cytometry. Background staining was determined in the absence of added first antibody. OVCAR-3, ES-2, and TOV-112D are previously identified ovarian cancer cell lines, CCD39SK is a skin fibroblast line, and TTB-6 is being tested to determine if it is of epithelial origin. The data are representative of a minimum of two independent experiments for each cell line

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Table 2 Class I MHC genotype of ovarian cancer lines

Tumor Line	Pathology	Molecular class I MHC typing		
		HLA-A	HLA-B	HLA-C
CAOV-3	Adenocarcinoma	6901	(49) or (4704, 4901)	07
CAOV-4	Adenocarcinoma	02	(15) or (15, 46)	03
COV413	Advanced ovarian cancer	02	07	07
ES-2	Clear cell carcinoma	03, 68	14 (65), 41	07, 08
OV-90	Adenocarcinoma	02	(4902, 58) or (50, 58)	06, 07
OVCAR-3	Adenocarcinoma	02, 29	07, 5805	07
SK-OV-3	Adenocarcinoma	03, 68	18, 35	04, 05
SW626	Adenocarcinoma	03	07	07
TOV-21G	Clear cell carcinoma	11, 26	(15, 40) or (40, 95)	02, 04
TOV-112D	Endometrioid carcinoma	03	14, 41	07, 08
TTB-6	Adenocarcinoma	(02, 68) or (02)	4037, 44	02, 07

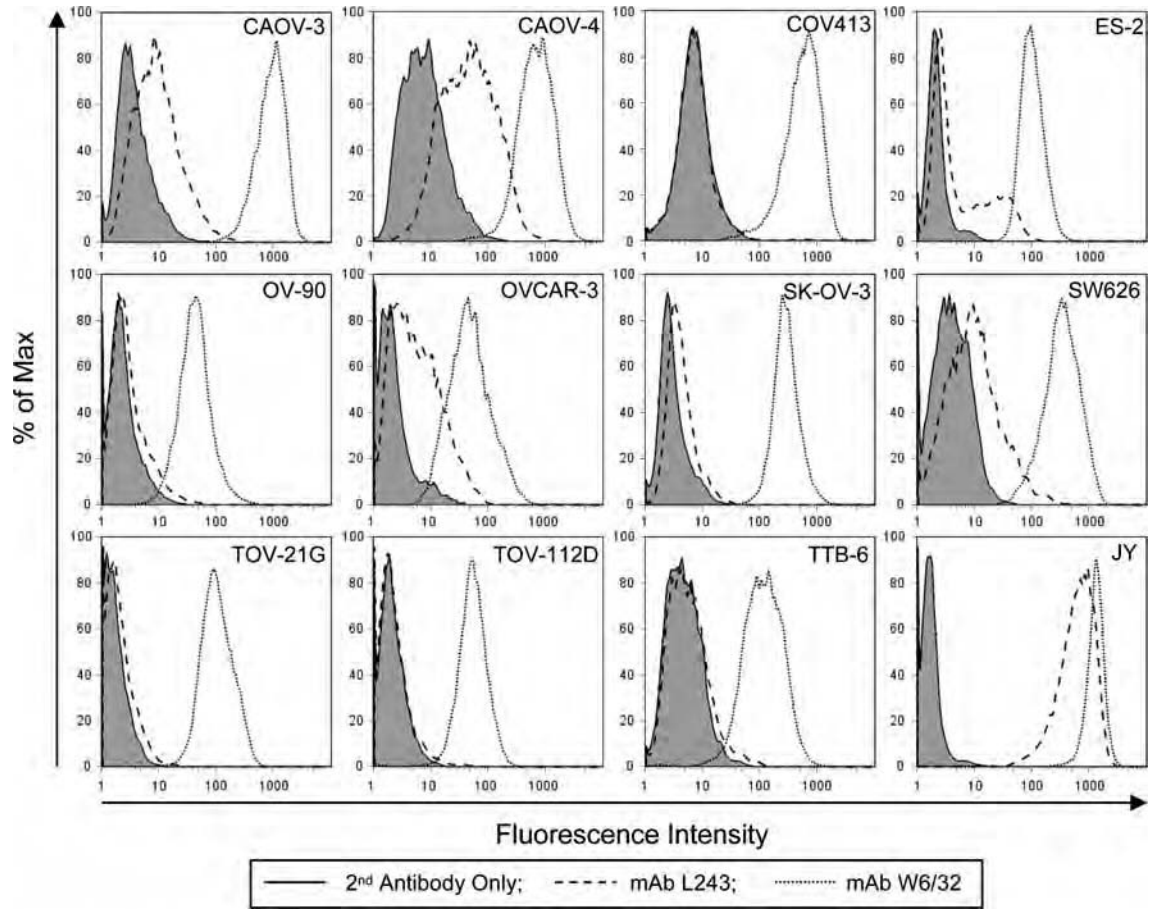

Fig. 2 Class I and class II MHC protein expression as determined by flow cytometry. Class I MHC proteins were detected using mAb W6/32 and class II MHC proteins were detected using mAb L243. Background staining was determined in the absence of added first antibody. JY is a B-LCL, which was used as a positive binding control for mAbs W6/32 and L243. The data are representative of a minimum of two independent experiments for each cell line

Tumor antigen expression

PCR was used to determine the mRNA expression levels of fourteen tumor antigens (Table 4). Each ovarian cancer line had a unique pattern of tumor antigen expression, and expressed between six and twelve of the tested antigens. The expression of the individual antigens among the cancer lines ranged from two to eleven positive lines for each of

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Table 3 Expression of class I MHC proteins on ovarian cancer lines

	Tumor cell lines													
	CAOV-3 (A69) [a]	CAOV-4 (A2)	COV413 (A2, B7)	ES-2 (A3, 68)	OV-90 (A2)	OVCAR-3 (A2, B7)	SK-OV-3 (A3, 68)	SW626 (A3, B7)	TOV-21G	TOV-112D (A3)	TTB-6 (A2, 68)	Controls		
												C1R-A2 (A2)	C1R-A3 (A3)	C1R-B7 (B7)
Experiment 1														
BB7.2 (A2, 69) [b]	**89.8** [c]			1.1	**13.2**	**8.6**	2.3		2.0	1.0		**247.8**	1.1	1.0
CR11-351 (A2,68, 69)	**62.6**			**12.5**	**13.6**	**8.7**	**22.8**		1.0	3.4		**301.7**	11.4	1.0
GAP-A3 (A3)	3.0			**33.8**	3.0	1.5	**42.3**		1.0	**26.0**		2.0	**105.7**	1.0
ME1-1.2 (B7, 27)	2.7			1.9	1.7	**14.8**	1.9		1.0	1.8		2.4	1.1	**199.8**
W6/32 (All class I)	**205.8**			**56.8**	**31.1**	**36.7**	**75.6**		**55.3**	**36.6**		**287.0**	**129.8**	**238.1**
2nd Only	2.0			1.0	1.2	1.3	1.5		1.0	1.0		1.0	1.0	1.0
Experiment 2														
BB7.2 (A2, 69)		**190.1**				**25.5**		9.4			**47.7**	**937.3**	3.5	3.2
CR11-351 (A2,68, 69)		**226.7**				**38.1**		64.4			**92.0**	**978.8**	33.9	2.1
GAP-A3 (A3)		7.8				4.2		**572.9**			4.0	4.2	**470.8**	3.3
ME1-1.2 (B7, 27)		27.8				**30.8**		**452.6**			4.8	7.9	2.7	**565.4**
W6/32 (All class I)		**502.9**				**88.5**		**1063.7**			**208.6**	**1107.0**	**668.0**	**1214.7**
2nd only		7.5				4.5		8.8			3.6	2.4	2.4	2.1

	Tumor cell lines											
	CAOV-3 (A69) [a]	CAOV-4 (A2)	COV413 (A2, B7)	ES-2 (A3, 68)	OV-90 (A2)	OVCAR-3 (A2, B7)	SK-OV-3 (A3, 68)	SW626 (A3, B7)	TOV-21G	TOV-112D (A3)	TTB-6 (A2, 68)	Controls
												JY (A2, B7)
Experiment 3												
BB7.2 (A2, 69)			**105.8**	1.6	**20.8**		2.2			1.5		**259.5**
GAP-A3 (A3)			7.9	**56.7**	3.9		**69.1**			**34.0**		1.3
ME1-1.2 (B7, 27)			**69.2**	6.8	2.0		2.0			1.4		**313.5**
W6/32 (All class I)			**217.9**	**116.3**	**49.2**		**181.0**			**47.2**		**809.3**
2nd only			7.0	1.2	1.6		1.7			1.2		1.0

Flow cytometry with antibodies directed against class I MHC molecules was performed as indicated in the Materials and Methods section

[a] The class I MHC molecules potentially expressed by the cell lines (see Table 2 for the complete molecular typing) and for which mAbs are available to confirm protein expression

[b] The specificity of the mAb is given in the parentheses

[c] Median fluorescence activity. Bolded values correspond to reactions expected to be positive if the corresponding class I MHC gene in the line is expressed. Data are representative of a minimum of two independent experiments

Table 4 Expression of tumor antigen genes in ovarian cancer cell lines

Antigen	Tumor cell lines											Antigen positive cell lines
	CAOV-3	CAOV-4	COV413	ES-2	OV-90	OVCAR-3	SK-OV-3	SW626	TOV-21G	TOV-112D	TTB-6	
MAGE-A1	–	–	–	+++	+++	–	+++	–	–	–	–	3
MAGE-A2	+	++	+	+	+	+	+	+	++	+	+	11
MAGE-A3	–	–	–	++++	++++	–	++	+	–	+	–	5
MAGE-A4	–	–	++	++	++	–	–	–	–	+	–	4
MAGE-A6	–	+	–	+++	+++	–	++	+	+	+	+	8
MAGE-A10	–	–	–	++	++	+++	–	–	–	–	–	3
MAGE-A12	+++	+++	+++	++++	++++	+++	+++	+++	+++	+++	+++	11
NY-ESO-1	++	–	++	++	+++	++	++	++	–	++	++	9
TAG-1	++	+	–	+	++	++++	+	++	+	–	+	9
TAG-2a	+	–	+	–	++	++++	–	++	–	–	++++	6
TAG-2b	–	–	–	–	–	++++	–	–	–	–	+++	2
TAG-2c	–	–	–	–	–	++++	–	+	–	–	++++	3
Her-2/neu	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	11
FBP	++++	++++	+++	+	++++	++++	++++	++++	++++	+++	++++	11
# of Expressed Antigens	7	6	7	11	12	10	9	10	6	8	10	

Gene expression was determined by PCR as indicated in the "Materials and methods". Data represent the average expression levels obtained using a minimum of two replicate experiments at each of 30 and 40 cycles of analysis. Data are reported as: (++++) easily visible at 30 cycles; (+++) weakly visible at 30 cycles, easily visible at 40 cycles; (++) not visible at 30 cycles, easily visible at 40 cycles; (+) not visible at 30 cycles, weakly visible at 40 cycles; and (–) not visible at 30 or 40 cycles

the antigens. The variability in expression occurred within the cancer/testis antigens, while Her-2/neu and FBP were found to be expressed in each line tested.

Antibodies recognizing CEA, Her-2/neu, FBP, and MAGE-A1 are available, and were used with flow cytometry to assess the expression of the respective molecules at the protein level (Fig. 3). CEA was expressed in OV-90 but not in any other ovarian cancer cell line. Her-2/neu was expressed at high levels in SK-OV-3, but no other ovarian cancer cell lines. FBP was expressed at elevated levels in CAOV-3, OV-90, SW626, and TTB-6, while MAGE-A1 was expressed at elevated levels in COV413, ES-2, SK-OV-3, TOV-21G, and TOV-112D.

TGF-β1, TGF-β2, TGF-β3, and IL-10 expression

The production of TGF-β1, TGF-β2, TGF-β3, and IL-10 was measured in supernatants collected from the ovarian cancer lines following 48 h of growth (Fig. 4). TGF-β exists in two forms, active and latent. Active TGF-β can be measured directly by ELISA, while the latent form must first be activated, and was done here by acid treatment. Total TGF-β is thus a measure of both pre-existing, active TGF-β, and newly activated TGF-β derived from the latent form of the protein.

Only low amounts of active TGF-β1 were measured in CAOV-3, COV413, and ES-2. Following acid activation, the total TGF-β1 measured from these same three cell lines was elevated above that endogenously present in FBS. The remaining ovarian cancer lines either did not produce, or only produced negligible amounts of TGF-β1. Active TGF-β2 was measured in all of the supernatants obtained from the ovarian cancer cell lines and ranged from about 15 to 100 pg/ml above that found in FBS. Substantial amounts of total TGF-β2 (>1,500 pg/ml) were found in COV413, OVCAR-3, SW626, and TOV-112D-derived supernatants, while lesser amounts (>300 pg/ml) were found in ES-2, OV-90, and SK-OV-3. Active and total TGF-β3 was either absent or present in only small amounts (<25 pg/ml). Only SW626 produced significant amounts of IL-10.

Discussion

When establishing new ovarian cancer cell lines it is important to determine that the line is of epithelial origin and not fibroblast origin, as the latter cell type can readily become established in a culture initially containing both cell types as is usually the case with patient samples. One characteristic of epithelial cells that can be used to distinguish them from other cell types is the expression of cytokeratins [35]. The mAb CAM5.2 [34], which recognizes cytokeratins 7 and 8 (Ck 7/8) [59] and the mAb NCL-5D3 [3], which recognizes cytokeratin 8, and to a lesser extent cytokeratins 18

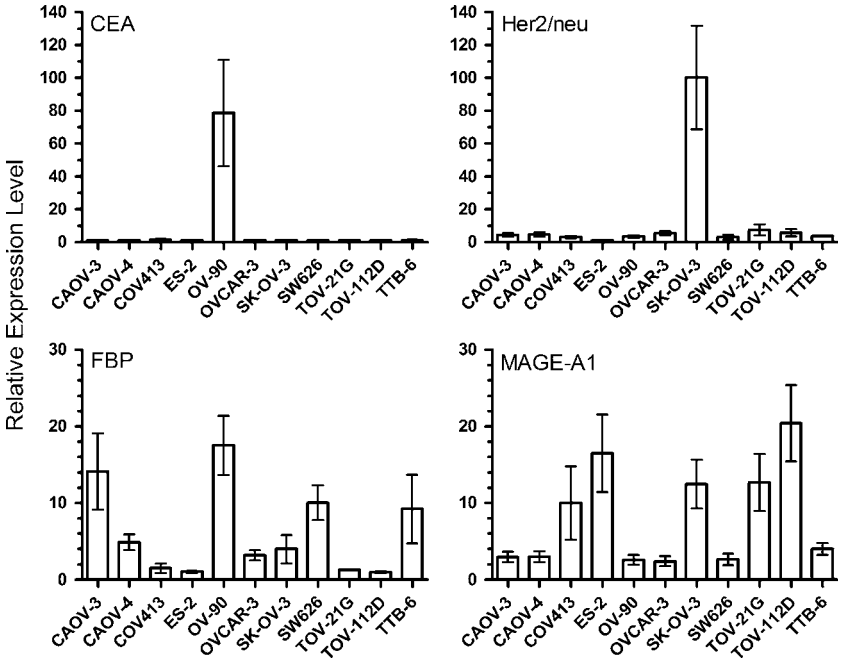

Fig. 3 Tumor antigen expression as determined by flow cytometry. mAb COL-1 (anti-CEA), mAb TA-1 (anti-Her-2/neu), and mAb Mov18/ZEL (anti-FBP) were used to stain unfixed cells, while mAb 3F257 (anti-MAGE-A1) was used to stain cells that had been fixed and permeabilized. Background staining was determined in the absence of added first antibody. Data are presented as the ratio of fluorescence activity obtained in the presence of both first and second antibody to that obtained in the presence of second antibody only, and are the average (±SD) of three independent experiments for each cell line

and 19 (Ck 8/18/19), have been shown to recognize ovarian cancer cells [3, 34, 48, 60]. In contrast, mAb AS02 recognizes CD90 on the surface of fibroblasts [53]. The newly described TTB-6 ovarian cancer cell line described here is recognized by mAb NCL-5D3 and not by mAb AS02 (Fig. 1), thus confirming that it is of epithelial origin.

In contrast to the other nine ovarian cancer cell lines tested here, both ES-2 and TOV-112D were not recognized by mAb NCL-5D3 (Ck 8/18/19 specific) (Fig. 1). TOV-112D was previously shown to be recognized by mAb CAM5.2 (Ck 7/8 specific) [48], which in combination with the present results, indicates the line expresses Ck 7, but not Ck 8/18/19. ES-2 was previously shown to be recognized by mAb OV-TL 12/30 (Ck 7 specific) but not by an antibody specific for Ck 8 [60], which in combination with the results presented here, indicates that the line expresses Ck 7 and not Ck 8/18/19. These results indicate that not all ovarian cancer cell lines are uniform in their expression of particular cytokeratins, and that multiple antibodies may be needed to accurately determine if a particular cell line expresses one or more cytokeratins.

The recognition of CD90 on TOV-112D by mAb AS02 suggests that the line is a fibroblast (Fig. 1), however, several lines of evidence argue against this interpretation. First, it has previously been demonstrated that mAb CAM5.2 binds to TOV-112D [48]. As indicated above, this result in combination with our own indicates that the cells express CK 7 associated with epithelial cells and not fibroblast. Second, the line expresses eight tumor antigens (Table 4), the expression of which is associated with tumor cells and not fibroblasts. Third, even if the small, CD90 negative population in TOV-112D represented epithelial cells and the large, CD90 positive population in TOV-112D represented fibroblasts, this could not be reconciled with the flow cytometry data. In these experiments, the entire TOV-112D population of cells is uniformly Her2/neu positive and MAGE-A1 positive, and the positive populations are clearly separated from the negative control. Thus, expression of Her2/neu and MAGE-A1 cannot be accounted for by a small, sub-population of cells, but rather reflects expression by all the cells in the population. Fourth, the small CD90 negative population was randomly observed in only two of five experiments, thus arguing against this population accounting for the expression of the tumor antigens. Taken as a whole, these results argue that TOV-112D is of epithelial origin, despite the fact that it expresses CD90. As mAb AS02 has been used in conjunction with magnetic beads to deplete cell cultures of fibroblasts [54], caution must be used to first ensure that the epithelial cell population does not also co-express CD90. It is difficult to accurately estimate how frequently ovarian cancer cell lines might express CD90 as the 95% confidence interval for the frequency based on a measurement of one positive line among eleven lines is 0.2–41.3%.

In order for the ovarian cancer lines to be useful in studies designed to determine the specificity of tumor reactive CTL it is necessary to know which class I MHC molecules the lines express. This question was addressed by a two-fold approach: first, by using molecular PCR typing to determine the class I MHC genotype of the cells (Table 2); and second, by assessing the surface expression of select class I MHC molecules for which mAbs are available (Table 3). The results of the PCR typing indicate that a minimum of two of the lines (COV413 and SW626) and

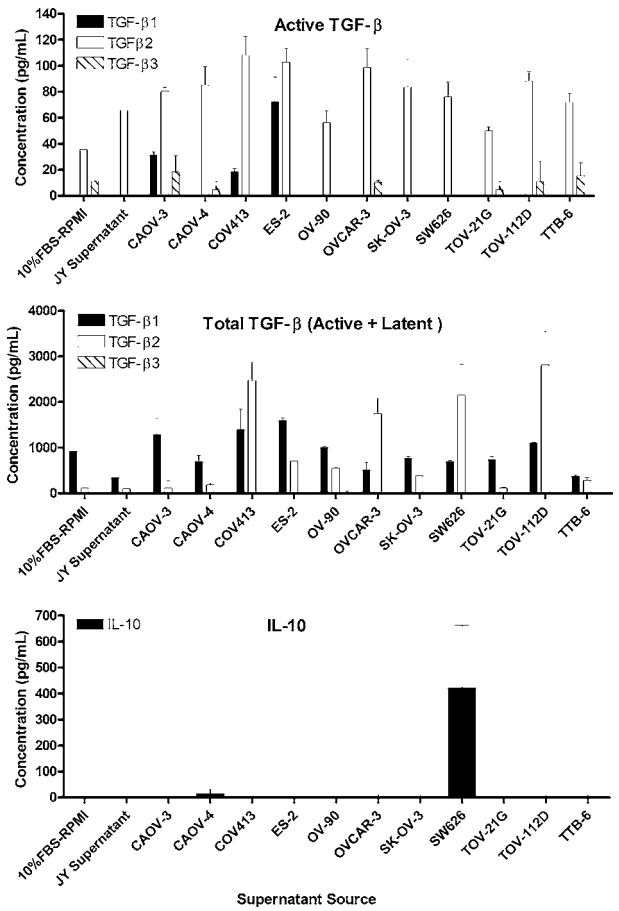

Fig. 4 TGF-β and IL-10 expression as determined by ELISA. Supernatants from ovarian cancer cell lines were obtained 48 h after 3×10^6 cells were added to T75 flasks in 8 ml of RPMI-10FBS. Total TGF-β was determined by acid-activation of the latent form of the cytokine. Cytokine concentrations were determined using antibody pairs for TGF-β1, TGF-β2, TGF-β3, and IL-10. Data are presented as the average (±SD) of two independent experiments

perhaps an additional two lines (CAOV-3 and CAOV-4) are either homozygous for expression of the HLA-A, -B, and -C alleles, or that they have undergone the deletion of a complete haplotype on one copy of chromosome 6. As the loss of class I and class II MHC expression through chromosomal deletions is a relatively frequent event in cancer cells [57], the loss of a haplotype is a likely explanation for this observation. Homozygous expression cannot be excluded, however, as typing of normal cells from the corresponding patients would be required and such material is not available.

The use of mAb W6/32, specific for an epitope present on all class I MHC molecules, confirmed that each of the ovarian cancer cell lines in comparison to B-LCL lines, expresses low to moderate levels of class I MHC molecules (Fig. 2; Table 3). The use of mAbs specific for particular class I MHC molecules allowed us to further confirm the expression of individual class I MHC molecules, again at levels that are low to moderate in comparison to levels found on B-LCL. This information is particularly informative when choosing ovarian cancer lines for use as stimulators or targets when stimulating or assessing the specificity of ovarian cancer-specific CTL. As part of this same analysis, several ovarian cancer cell lines were also shown to express class II MHC molecules, thus indicating that they may have the ability to stimulate class II MHC restricted responses.

To be of value in defining the antigens recognized by ovarian cancer-specific CTL it is also important to have a panel of tumor cell lines that have been characterized for antigen expression. The eleven ovarian cancer cell lines studied here were tested for the expression of twelve cancer/testis antigens, Her-2/neu, and FBP (Table 4; Fig. 3). As determined by PCR, each of the tested antigens was expressed in between three and all eleven of eleven lines tested. When antigen expression is assessed on individual ovarian cancer cell lines, it is seen that each line expresses between six and twelve of the fourteen studied antigens.

The availability of antibodies to some of the tested antigens allowed for the further assessment of the antigens at the protein level (Fig. 3). Her-2/neu was clearly over-expressed in SK-OV-3 (100.2-fold over background), and is present at 3.0- to 7.4-fold over background in all the remaining lines with the exception of ES-2. These results are consistent with a previous report demonstrating that SK-OV-3, TOV-21G, and TOV-112D express Her-2/neu as demonstrated by immunohistochemistry [48]. Likewise, FBP was clearly over-expressed at the protein level in CAOV-3 (14.1-fold), OV90 (17.5-fold), SW626 (10.1-fold), and TTB-6 (9.2-fold), and to a lesser extent in CAOV-4 (4.9-fold), OVCAR-3 (3.2-fold), and SK-OV-3 (4.0-fold). As with Her-2/neu, a positive PCR at 30 and 40 cycles was poorly predictive of total protein. These results indicate that caution must be used when assessing antigen expression solely on the basis of the strength of the PCR signal. The lack of a strong correlation between PCR reactivity and antibody reactivity could be due to the fact that relatively high mRNA expression saturates the PCR signal even at 30 cycles of amplification, gene-specific mutations preclude the ability of the proteins to be expressed, or that additional factors regulate protein expression.

The results show that the anti-MAGE-A1 antibody bound to three lines (COV413, TOV-21G, and TOV-112D) that were PCR negative for the MAGE-A1 gene (Table 4; Fig. 3). The most likely explanation for binding to MAGE-A1 negative cell lines is cross-reactive binding on other MAGE-A proteins as has been reported for other MAGE-specific antibodies including 57B and 6C1 [50]. An analysis of the results does not readily indicate another MAGE-A gene product that might be recognized. As we have used PCR to only test for the seven most prevalent of the eleven

expressed MAGE-A genes, the possibility remains that additional, less prevalent MAGE-A genes are expressed in the cell lines and recognized by the antibody. It is also possible that only a small fraction of a line expresses a particular gene when that line is found to be positive by PCR, and that the antibody binding results are an accurate assessment of protein expression for those lines.

As CEA expression can be detected by immunohistochemistry in a low percentage of ovarian cancer samples [31], each of the ovarian cancer cell lines was also tested for CEA expression. As determined by flow cytometry, only OV-90 expressed CEA. Interestingly, SW626 did not express CEA. Although SW626 was originally reported to be an ovarian cancer cell line [18], a more recent report provides evidence that it may actually be of colonic origin [19]. The expression of FBP and the lack of expression of CEA is, however, consistent with the line being of ovarian origin.

Both primary and cultured ovarian cancer cells have been shown to express TGF-β [5, 21, 37, 62]. TGF-β inhibits the in vitro generation of CTL [36, 49, 63] and blocks in vivo tumor immunosurveillance [61]. To the extent that cell lines in our ovarian cancer cell line panel will be used in an attempt to stimulate ovarian-specific CTL, it is important to know whether or not any of the ovarian cancer cell lines in our panel express suppressive cytokines. Some, but not all of the cell lines, produced TGF-β1 and/or TGF-β2, but not TGF-β3. Four of the lines (COV413, OVCAR-3, SW626, and TOV-112D) produced substantial amounts of the latent form of TGF-β2 as none of them produced more than 100 pg/ml of active TGF-β2, and the total amount measured for each was >1,500 pg/ml. These concentrations may be biologically significant as TGF-β1 and -β2 at concentrations greater than about 500 ng/ml have been shown to suppress the in vitro generation of CTL [1, 36, 49, 63].

IL-10 is infrequently expressed in ovarian cancer cell lines [7, 22, 62], is present in the ascites of patients with ovarian cancer [22, 55], and is associated with the suppression of T cell responses [2]. Only one ovarian cancer cell line (SW626) of the eleven tested here expressed appreciable amounts of IL-10, an amount that was previously shown to be biologically significant in blocking anti-CD3-induced T cell proliferation [9].

The ovarian cancer cell lines characterized here all express class I MHC molecules and a variety of tumor antigens. Some, but not all of the lines, also express immunosuppressive cytokines. This comprehensive analysis will serve to increase the utility of these cell lines in the characterization of antigens recognized by ovarian cancer-specific CTL.

Acknowledgments This work was supported by grant W81XWH-05-1-0012 from the United States Department of Defense to K. T. Hogan.

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APPENDIX 1

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The TAG Family of Cancer/Testis Antigens is Widely Expressed in a Variety of Malignancies and Gives Rise to HLA-A2–Restricted Epitopes

Sara J. Adair, Tiffany M. Carr, Mitsú J. Fink, Craig L. Slingluff, Jr, and Kevin T. Hogan

Summary: The TAG-1, TAG-2a, TAG-2b, and TAG-2c cancer/testis genes, known to be expressed in an unusually high percentage of melanoma cell lines, are shown here to be expressed in a variety of tumor lines of diverse histologic type, including cancers of the brain, breast, colon, lung, ovary, pharynx, and tongue. The genes are also expressed in fresh, uncultured melanoma, and ovarian cancer cells. Epitope prediction algorithms were used to identify potential HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 epitopes, and these potential epitopes were tested for their ability to stimulate a peptide-specific cytotoxic T lymphocyte response using lymphocytes from healthy donors. Two HLA-A2–restricted epitopes (SLGWLFLLL and LLLRLECNV) were identified using this approach. Cytotoxic T lymphocytes specific for each of these peptides were capable of recognizing tumor cells expressing both the corresponding class I major histocompatibility complex encoded molecule and the TAG genes. These results indicate that TAG-derived peptides may be good components of a therapeutic vaccine designed to target melanoma and a variety of epithelial cell-derived malignancies.

Key Words: cancer/testis antigen, CTL, epitope, immunotherapy

(*J Immunother* 2008;31:7–17)

Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity and cytokine secretion have emerged as major mechanisms by which tumor growth is controlled by the mammalian immune system.[1] Vaccination of mice with immunogenic peptides has been shown to control tumor growth in both therapeutic and prophylactic models of cancer.[2,3] Vaccine trials have begun in humans, with most efforts using antigenic peptides known to bind to class I major histocompatibility complex (MHC) molecules, although class II MHC molecule binding peptides are also being tested.[4–7] Although a substantial number of peptides have been discovered that can be used for the treatment of melanoma, there are relatively fewer peptide antigens that can be used for the treatment of other malignancies.[8–10] Identification of additional peptide antigens would expand the number of malignancies that are amenable to vaccine-mediated therapy, and the use of a large number of peptides in a vaccine would minimize the impact of antigen loss variants that arise in the presence of immunoselection.[11–14]

The categories of proteins giving rise to the tumor antigens recognized by CTL include cancer/testis antigens, differentiation antigens, mutated gene products, widely expressed proteins, and viral proteins.[15–17] Cancer/testis antigens are particularly attractive candidates for use in tumor vaccines, as these antigens are only expressed in the testis and occasionally the placenta, which are both immunologically privileged sites.[18] A consequence of this pattern of expression is that the peripheral CTL are not rendered tolerant to cancer/testis antigens and can thus recognize the antigens when they are expressed on tumor cells. The cancer/testis antigen family now contains a wide variety of proteins, prototypic members of which are exemplified by the MAGE[19] and NY-ESO-1[20] antigens. More recently, we have identified multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) of a gene coding for a new cancer/testis antigen.[21]

The TAG-1, TAG-2a, TAG-2b, and TAG-2c genes were previously shown to be expressed in almost 90% of 32 melanoma lines tested.[21] Unlike most cancer/testis antigens, which are rarely expressed in leukemia or myeloma cells, the TAG genes are also expressed in K562, a myelogenous leukemia, and they are homologous with chronic myelogenous leukemia-derived clones in the human EST database.[21] Importantly, TAG is naturally immunogenic as the TAG-derived peptide RLSNRLLLR was recognized by HLA-A3–restricted CTL obtained from a melanoma patient.[21,22] We have expanded on this work in the present study by demonstrating that the TAG gene is expressed in a variety of epithelial cell-derived

Received for publication July 11, 2007; accepted August 27, 2007.
From the Department of Surgery and the Human Immune Therapy Center, University of Virginia, Charlottesville, VA.
Supported by grants from the Department of Defense (W81XWH-05-1-0012 to K. T. Hogan) and the National Institutes of Health (R01CA90815 to K. T. Hogan).
Financial Disclosure: Dr Kevin T. Hogan declares that the University of Virginia Patent Foundation has filed a patent application on the peptides described in this publication, and that Dr Kevin T. Hogan is the inventor on the patent application. The remaining authors have declared that there are no financial conflicts of interest.
Reprints: Kevin T. Hogan, Department of Surgery, University of Virginia, Charlottesville, Box 801359, VA 22908 (e-mail: kh6s@virginia.edu).

tumors and by identifying additional TAG-derived peptides that elicit tumor-reactive CTL responses.

MATERIALS AND METHODS

Growth Medium

RPMI-1640 supplemented with 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (complete RPMI) served as the base medium. Base medium was supplemented with 10% fetal bovine serum (RPMI-10FBS) or 10% human serum (RPMI-10HS). For the growth of some tumor lines, RPMI-10FBS was additionally supplemented with 1 mM of sodium pyruvate, 5 ng/mL of epidermal growth factor, 10 μg/mL of transferrin, and 7.3 μg/mL of insulin (RPMI-10FBS-PETI).

Cell Lines

The melanoma lines AVL3-Mel, DM6, DM13, DM93, DM281, SK-Mel-5, VMM5A, VMM12, and VMM39 were available from our laboratory. Melanoma lines indicated as TAG^+ in the cytotoxicity experiments described below were all previously shown to be strongly positive for TAG-1 and TAG-2a mRNA expression as determined by polymerase chain reaction (PCR) amplification.[21] The brain cancer lines SW-1088, T98G, U-87MG, and U-373MG were obtained from the ATCC (Manassas, VA). The breast cancer lines MCF7, MDA-MB-453, MDA-MB-468, and SK-BR-3 were obtained from the ATCC; the breast cancer lines VAB5-A, BRC-173, and BRC-751 were established at the University of Virginia. The colon cancer lines HT-29, LS174T, and SW480 were obtained from the ATCC; the colon cancer line VCR-8 was established at the University of Virginia. The lung cancer lines Calu-1, SK-LU-1, and SK-MES-1 were obtained from the ATCC; the lung cancer lines TTB-250, VBT-2, VLU-6, VLU-13, VLU-18, and VLU-19 were established at the University of Virginia. The ovarian cancer lines CAOV-3, CAOV-4, ES-2, OV-90, OVCAR3, SK-OV-3, SW626, TOV-21G, and TOV-112D were obtained from the ATCC. The ovarian cancer cell line TTB-6 was established at the University of Virginia, and the ovarian cancer cell line COV413 was obtained from Dr Angela Zarling (University of Virginia). The squamous cell carcinomas of pharyngeal (FaDu), tongue (SCC4), and cervical (SiHa) origins were obtained from the ATCC. The cancer lines were maintained in RPMI-10FBS or RPMI-10FBS-PETI.

The B-lymphoblastoid cell lines (B-LCL) JY, MST, and T2 were maintained in RPMI-10FBS. C1R-A2, C1R-A3, and C1R-B7 were maintained in RPMI-10FBS supplemented with 300 μg/mL G418.

Patient Material

Cryopreserved tumor digest was obtained from the Tissue Procurement Facility at the University of Virginia. The tissue was obtained in an anonymized fashion and in accordance with established Institutional Review Board protocols.

PCR

Total RNA was prepared from 2 to 10×10^6 cells using the RNeasy Mini kit (Qiagen, Valencia, CA) as per the kit instructions. RNA was quantified by absorbance at 260 nm. Total RNA was converted to cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Previously designed primers were used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1361, 1362; 598 bp), TAG-1 (A52, C723; 672 bp), TAG-2a (A73, E600; 528 bp), TAG-2b (A73.92, F473; 401 bp), and TAG-2c (A73, G608; 536 bp).[21] PCR was performed on 250 ng of cDNA using Platinum Taq High Fidelity (Invitrogen). The PCR mixes were heated to 94°C for 2 minutes, 30 and 40 cycles of amplification were performed, and a final extension completed at 68°C for 5 minutes. When amplifying the TAG genes, the 30 and 40 cycles consisted of 94°C for 30 seconds, 62°C for 30 seconds, and 68°C for 60 seconds. When the GAPDH gene was amplified, the 30 cycles consisted of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 seconds. The PCR products were visualized on ethidium bromide-stained agarose gels.

Epitope Prediction

The TAG-1 and TAG-2 genes can each be potentially translated into 3 different isoforms (TAG-1α, TAG-1β, TAG-1γ, TAG-2α, TAG-2β, and TAG-2γ), with the putative α, β, and γ forms differing in the length at the N-terminal end of the protein. For the purposes of epitope prediction, the largest isoforms of each, TAG-1α and TAG-2α, were used and are referred to herein as TAG-1 and TAG-2.

The SYFPEITHI (www.syfpeithi.de)[23] and Parker (bimas.cit.nih.gov/molbio/hla_bind)[24] epitope prediction algorithms were used to identify peptides that have a high predicted binding affinity for HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8. The peptides were synthesized (New England Peptide Inc, Gardner, MA) and resuspended at 2 to 10 μg/mL in 100% dimethyl sulfoxide.

Peripheral Blood Mononuclear Cells

The buffy coat fraction from a unit of blood was obtained as a byproduct of voluntary blood donations by healthy individuals (Virginia Blood Services, Richmond, VA). Peripheral blood mononuclear cells (PBMC) were obtained after centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) and washed twice. Monocytes were enriched from the PBMC by adherence to tissue culture flasks for 2 hours and were subsequently used for the generation of dendritic cells. The plastic nonadherent cells were used as peripheral blood lymphocytes (PBL) and were cryopreserved until use.

Class I MHC Gene Typing

DNA was obtained from 5×10^6 PBMC using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Class I MHC typing was carried out

using the Micro SSP Generic HLA Class I typing tray SSP1L (One Lambda Inc, Canoga Park, CA).

Dendritic Cell Preparation

Dendritic cells were prepared using a modification of a previously published protocol.[25] In brief, adherent monocytes were incubated for 6 to 8 days in the presence of 800 U/mL granalocyte macrophage colony-stimulating factor and 500 U/mL interleukin (IL)-4 to produce immature dendritic cells. The nonadherent, immature dendritic cells were then incubated in the presence of 800 U/mL granalocyte macrophage colony-stimulating factor, 500 U/mL IL-4, 10 ng/mL IL-1β, 1,000 U/mL IL-6, 10 ng/mL tumor necrosis factor-α, and 1 μg/mL prostaglandin E2. Mature dendritic cells (mDC) were obtained as nonadherent cells after 3 to 4 days of incubation. mDC were incubated with peptide (40 μg/mL) and β_2-microglobulin (3 μg/mL) for 2 hours at room temperature. The peptide-pulsed mDC were irradiated (3500 Rad) and washed once to remove free peptide. The cells were then used immediately for CTL stimulation or were cryopreserved for future stimulations.

Stimulation of Peptide-specific CTL

CTL were stimulated using a modification of the protocol of Lu and Celis.[26,27] Equal volumes of PBL (2×10^6 cells/mL) were mixed with autologous, peptide-pulsed mDC (1×10^5 cells/mL) to give a responder to stimulator ratio of 20:1 in RPMI-10HS supplemented with 10 ng/mL IL-7. Wells (generally 48) on a 48-well plate were seeded with 0.5 mL of the mixed responder/stimulator cells. One day after the initial priming, IL-10 at a final concentration of 10 ng/mL was added to each well. The cultures were restimulated every 7 days. For secondary stimulations, peptide-pulsed stimulator cells were added to each well in 0.5 mL RPMI-10HS. IL-10 (10 ng/mL) was added 1 day later, and IL-2 at a final concentration of 10 Cetus U/mL was added 2 days later. Two to 3 days later, IL-2 (10 Cetus U/mL) was added again to each culture. Tertiary and later stimulations were performed in a similar fashion except that IL-2 was the only added cytokine. For all stimulations, 2.5×10^4 mDC were used as the stimulator cells in the restimulations until they were depleted, after which 1×10^6 autologous PBL or 2.5×10^5 to 5.0×10^5 B-LCL matched for the class I MHC molecule of interest were used. T-cell cultures showing activity against peptide-pulsed targets in a screening assay were expanded with anti-CD3.[28]

Cytotoxicity Assays

A standard 4 hour ^{51}Cr-release assay was used and modified as indicated below for the indicated analyses.[29] Target cells were labeled with 100 μCi $Na_2\ ^{51}CrO_4$ for 2 hours and then washed. The ^{51}Cr-labeled cells were then incubated with peptide (10 μg/mL) for 1 hour at room temperature, washed, and added to wells containing CTL on a 96-well plate at 2000 cells/well. Maximal ^{51}Cr-release was determined by incubating labeled target cells in the presence of 3% NP-40, and spontaneous ^{51}Cr-release was obtained by incubating with assay medium alone. Counts per minute (CPM) present in the collected supernatants were measured using a Wallac WIZARD automatic gamma counter (Perkin Elmer, Downers Grove, IL). The percent specific release was calculated as: % specific ^{51}Cr-release = 100 × (ER-SR)/(MR-SR), where ER = CPM experimental release, SR = CPM spontaneous release, and MR = CPM maximal release.

Cytotoxicity Screening Assay

T-cell cultures were tested for cytotoxic activity against peptide-pulsed cells 5 to 6 days after the fourth stimulation. Cells from 4 to 6 randomly selected wells from each 48-well plate to be tested were counted and averaged to obtain a mean effector cell concentration. Average effector-to-target (E:T) ratios of 40:1 and 10:1 were tested in duplicate for each culture. In some experiments, unlabeled K562 cells were added at an unlabeled-to-labeled target ratio of 20:1 to decrease nonspecific killing attributable to natural killer cells.

CTL Specificity Analysis

^{51}Cr-labeled tumor cells and peptide-pulsed B-LCL were used as targets. CTL cultures were counted and resuspended at a concentration giving an initial E:T ratio which generally ranged from 20:1 to 80:1, with subsequent 1:2 dilutions used to give 4 E:T ratios for each CTL being analyzed.

Peptide Titration

Peptides, starting at 10 μg/mL, were diluted through a 10-fold dilution series, after which ^{51}Cr-labeled targets were added for 1 hour at room temperature. The target cells were then resuspended at 20,000 cells/mL and added to the appropriate wells of the 96-well plates in 100 μL aliquots. Effector cells were then added at 100 μL/well to give the desired E:T ratio.

Cold Target Inhibition

Cold targets, starting at a 60:1 cold-to-hot (C:H) ratio were added in a final volume of 50 μL per well. Cold target cells, when appropriate, were first incubated with peptide (0.1 to 10 μg/mL depending on avidity) for 1 hour at room temperature. Before plating, peptide-pulsed cold target cells were washed with 10 × volume of RPMI 1640 containing 1% FBS. Effector cells, in a volume of 50 μL/well, were then added. The 96-well plates were then centrifuged at 800 rpm for 2 minutes at room temperature and incubated at 37°C for 1 hour. After the incubation, ^{51}Cr-labeled targets were added in 100 μL aliquots. Each cell line used in the assay was also evaluated in triplicate, without cold targets, at the appropriate E:T ratios.

Human Subjects

All research involving human subjects and human tissues was approved by the University of Virginia Institutional Review Board in accordance with an assurance filed with and approved by the Department of Health and Human Services.

RESULTS

Expression of TAG Genes in Established Tumor Lines

The TAG genes were previously shown to be expressed in a large percentage of melanoma cell lines.[21] To determine if the TAG genes are expressed in other cancers, a broad survey was taken using established tumor lines of nonmelanocytic origin. Brain tumors, which share an ectodermal origin with melanocytes, expressed the TAG-1, TAG-2a, and TAG-2c genes, but not the TAG-2b gene (Table 1). Tumor lines of epithelial cell origin including breast, colon, lung, ovarian, pharyngeal, tongue, and cervical origin were also tested for expression of the TAG genes (Table 1). With the exception of the pharyngeal, tongue, and cervical tumor lines for which only 1 cell line each was tested, each of the TAG genes was expressed in at least 1 line of each cancer type. As with melanoma,[21] TAG-1 was the most frequently expressed TAG gene, whereas TAG-2b was the least frequently expressed TAG gene. By performing the PCR amplification at both 30 and 40 cycles, it was

TABLE 1. Expression of TAG Genes in Established Tumor Lines*

Tumor Tissue Origin and Type	TAG-1	TAG-2a	TAG-2b	TAG-2c
Tumors of neural origin				
Brain	75% (19%-99%)	50% (7%-93%)	0% (0%-60%)	50% (7%-93%)
SW-1088	+	+	−	−
T98G	+	+	−	+
U-87MG	+	−	−	+
U-373MG	−	−	−	−
Tumors of epithelial origin				
Breast	71% (29%-96%)	14% (4%-58%)	14% (4%-58%)	14% (4%-58%)
BRC-173	−	−	−	−
BRC-751	+	−	−	−
MCF7	−	−	−	−
MDA-MB-453	+	−	−	−
MDA-MB-468	+ + +	+ + +	+ +	+ +
SK-BR-3	+	−	−	−
VAB5-A	+	−	−	−
Colon	100% (40%-100%)	50% (7%-93%)	25% (6%-81%)	50% (7%-93%)
HT-29	+	−	−	−
LS174T	+	+	−	+
SW480	+	−	+	−
VCR-8	+ + +	+	−	+
Lung	67% (30%-93%)	78% (40%-97%)	44 (14%-79%)	44 (14%-79%)
Calu-1	−	+	−	−
SK-LU-1	+	−	−	−
SK-MES-1	+ +	+ +	+	+
TTB-250	−	+	−	−
VBT-2	+	+	−	−
VLU-6	−	−	−	−
VLU-13	+ + +	+ + +	+ + +	+ + +
VLU-18	+ + +	+ + +	+ +	+ +
VLU-19	+ +	+	+	+
Ovarian	82% (48%-98%)	55% (23%-83%)	18% (23%-52%)	27% (6%-61%)
CAOV-3	+	+	−	−
CAOV-4	+	−	−	−
COV413	−	+	−	−
ES-2	+	−	−	−
OV-90	+	+	−	−
OVCAR3	+ + +	+ + +	+ +	+ + +
SK-OV-3	+	−	−	−
SW626	+	+	−	+
TOV-21G	+	−	−	−
TOV-112D	−	−	−	−
TTB-6	+ + +	+ + +	+ +	+ + +
Pharyngeal	100% (2%-100%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
FaDu	+	−	−	−
Tongue	100% (2%-100%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
SCC4	+	−	−	−
Cervical	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
SiHa	−	−	−	−

*PCR was performed as described in the Materials and Methods. The PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: + + +, product was easily visualized after 30 cycles of amplification; + +, product was weakly visible at 30 cycles and easily visible after 40 cycles of amplification; +, product was only visible after 40 cycles of amplification; −, product was not visualized after 40 cycles of amplification. The percentage positive with 95% confidence intervals is given for each tumor type. Each sample exhibited a strong signal when GAPDH was PCR amplified.

also possible to categorize the expression levels in individual cell lines. The data demonstrate that the TAG genes are strongly expressed in some tumor lines, whereas weakly expressed in others (Table 1). Because a strong positive amplification signal of the GAPDH gene was obtained for each of the cDNA samples (data not shown), the lack of TAG gene amplification for a given sample cannot be attributed to the quality of the cDNA preparation.

Expression of TAG Genes in Uncultured Melanoma and Ovarian Carcinoma Cells

It was previously shown that the TAG genes were expressed in the uncultured melanoma cells from which the VMM18 melanoma line was established and from which the TAG genes were originally cloned and identified, thus demonstrating that the expression of the genes is not an artifact of in vitro cell culture.[21] That work has been extended here by showing that the TAG genes are expressed in a relatively high frequency of uncultured melanomas and ovarian carcinomas (Table 2). As with the tumor lines, TAG-1 is the most frequently expressed and TAG-2b is the least frequently expressed TAG gene.

Prediction of Class I MHC Binding Peptides From TAG-1 and TAG-2

The SYFPEITHI[23] and Parker et al[24] epitope prediction algorithms were used to predict HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 binding peptides from the TAG-1 and TAG-2 proteins (Table 3). The top 5 peptides predicted by each algorithm were reviewed and those peptides not containing canonical anchor residues were eliminated. The remaining 2 to 5 top ranked peptides were then selected for synthesis and testing. The peptide RLSNRLLLR was predicted to bind HLA-A3 (ranking in the top 1 to 4 scores for TAG-1 and TAG-2), but was not chosen for study as we previously demonstrated that it is recognized by a tumor-reactive CTL line that naturally developed in a melanoma patient.[21]

Generation of CTL Specific for TAG-derived Peptides

Each peptide listed in Table 4 was synthesized and tested for its ability to prime a peptide-specific CTL response using PBL obtained from 3 to 9 healthy donors. PBL were stimulated with peptide-pulsed, autologous mDC, generally in 48 individual microcultures per donor. After the fourth restimulation, the individual cultures were tested for reactivity with peptide-pulsed target cells in a ^{51}Cr-release assay. Cultures, with killing that was more than 20% above that found on the target cells not pulsed with peptide, were selected for additional characterization. Initially, individual microcultures were stimulated with only a single peptide, and these cultures identified 2 peptides for further study, including the HLA-A2–restricted peptide SLGWLFLLL and the HLA-B8–restricted peptide LSRLSNRLL (Table 4). Selected cultures reactive with SLGWLFLLL and LSRLSNRLL were expanded with anti-CD3 antibody for further analysis. The reactivities of 6 SLGWLFLLL cultures are shown in Figure 1. The culture reactive with the LSRLSNRLL peptide lost its peptide-specificity after expansion, and although it recognized tumor, it was not studied further.

TABLE 2. Expression of TAG Genes in Uncultured Melanoma and Ovarian Carcinoma Cells

	Gene Expression Pattern*			
Sample Numbers	**TAG-1**	**TAG-2a**	**TAG-2b**	**TAG-2c**
Melanoma				
204, 415, 7719	++	++	+	++
956	++	++	+	+
2241	++	++	–	++
8062	++	+	–	–
3540	+	+	+	+
1302	+	+	–	–
278, 550, 1435, 2348, 8353	+	–	–	–
123, 243, 482, 509, 2201, 4479, 8326, 8542, 8899	–	–	–	–
Total positive	13/22	8/22	5/22	6/22
% positive	59%	36%	23%	27%
95% confidence interval	36%-79%	17%-59%	8%-45%	11%-50%
Ovarian carcinoma				
6, 519	++	++	+	++
144, 632	+	+	–	–
117, 121, 189, 1006	+	–	–	–
1130	–	+	–	–
29, 94, 125, 136, 185, 212, 227, 258, 546, 567, 572, 834, 1288, 3883	–	–	–	–
Total positive	8/23	5/23	2/23	2/23
% positive	35%	22%	9%	9%
95% confidence interval	16-57%	8-44%	1-28%	1-28%

*PCR was performed as described in the Materials and Methods. Following 30 rounds of amplification the PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: (++) product was easily visualized; (+) product could be visualized, but the band was very light; (–) product was not visible. Each sample exhibited a strong signal when GAPDH was PCR amplified.

TABLE 3. Test Peptides From TAG Used for In Vitro CTL Priming

Class I MHC Binding Protein*	Peptide Sequence†	Residue Numbers‡	Presence in TAG-1 or TAG-2	Parker Score§	SYFPEITHI Score‖
HLA-A1	ESERGLPAS	32-40	1, 2	0.27	16
	NLEPLVSRD	64-72	1, 2	0.90	16
	SRDPPASAS	70-78	1, 2	0.25	17
HLA-A2	TLSRLSNRL	41-49	1, 2	21.4	22
	LLLRLECNV	49-57	1, 2	487.5	25
	SLGWLFLLL	78-86	1	40.6	24
	FLLLLNSTT	83-91	1	126.8	20
HLA-A3	GLPASTLSR	36-44	1, 2	24.0	21
	LLLLNSTTK	84-92	1	30.0	28
HLA-B7	LPAQEGAPT	1-9	1, 2	2.0	20
	VQRRAEGLL	10-18	1, 2	40.0	12
	LPASTLSRL	37-45	1, 2	80.0	21
	LSRLSNRLL	42-50	1, 2	40.0	12
	DPPASASLF	72-80	2	0.4	11
HLA-B8	TVQRRAEGL	9-17	1, 2	4.0	18
	VQRRAEGLL	10-18	1, 2	1.2	17
	LSRLSNRLL	42-50	1, 2	4.0	18

*Antigenic peptides from TAG-1 and TAG-2 were predicted based on the predicted ability of the peptides to bind to the indicated class I MHC molecule.
†Underscored residues correspond to canonical amino acid residues typically found at that position of a peptide binding to the respective class I MHC molecule.
‡Position of the peptide within the linear sequence of TAG-1 and TAG-2.
§Score obtained from predictive algorithm of Parker et al.[24]
‖Score obtained from the predictive algorithm of Rammensee et al.[23]

Screening individual test peptides for their ability to stimulate a CTL response is an inherently time and resource intensive endeavor. To determine the feasibility of screening multiple peptides simultaneously, PBL of 1 of the donors reactive against the SLGWLFLLL peptide in association with HLA-A2 was also stimulated with a mix of 4 peptides, including SLGWLFLLL. Because of limitations on the number of responder cells in each microculture, the initial screening was performed against targets pulsed with all 4 peptides, and this yielded positive cultures (Table 4). Cultures with cytotoxic reactivity were then expanded with anti-CD3 and tested against targets individually pulsed with each of the peptides used in the stimulation. Not only was a response against SLGWLFLLL in the mix detected, but a response was also measured against LLLRLECNV in association with HLA-A2, thus validating that multiple peptides can be tested simultaneously for their ability to induce a CTL response (Table 4; Figs. 2A, C). A similar peptide mix lacking the SLGWLFLLL peptide was also used to identify another LLLRLECNV reactive culture in an additional donor (Table 4). Likewise, a mix of HLA-B7 peptides led to the identification of LPAQEGAPT as a candidate epitope (Table 4; Figs. 2B, D). Although LPAQEGAPT-reactive CTL were shown to recognize tumor (data not shown), their specificity could not be confirmed in cold target inhibition experiments and results with this peptide are not discussed further.

TABLE 4. Summary of CTL Reactivity Against Stimulating Peptides

Class I MHC Binding Protein	Stimulating Peptide(s)*	No. Positive Cultures Obtained/No. Cultures Initiated†	Specificity of Obtained CTL‡
HLA-A1	SRDPPASAS	0/6	NT
HLA-A1	ESERGLPAS, NLEPLVSRD	0/6	NT
HLA-A2	SLGWLFLLL	7/9	SLGWLFLLL
HLA-A2	SLGWLFLLL, TLSRLSNRL, LLLRLECNV, FLLLLNSTT	1/1	SLGWLFLLL, LLLRLECNV
HLA-A2	TLSRLSNRL, LLLRLECNV, FLLLLNSTT	1/5	LLLRLECNV
HLA-A3	GLPASTLSR	0/6	NT
HLA-A3	LLLLNSTTK	0/6	NT
HLA-B7	LPASTLSRL	0/6	NT
HLA-B7	LPAQEGAPT, VQRRAEGLL, LSRLSNRLL, DPPASASLF	2/3	LPAQEGAPT
HLA-B8	LSRLSNRLL	1/9	LSRLSNRLL
HLA-B8	TVQRRAEGL, VQRRAEGLL	0/6	NT

*Peptide priming of CTL was performed as indicated in the Materials and Methods, and was carried out with either a single peptide or a pool of 2 to 4 peptides as indicated.
†A culture was considered positive if the responder cells in at least 1 well from a given donor recognized target cells pulsed with the corresponding peptide(s) in both initial screening assays.
‡Initial screening for positive cultures used target cells pulsed with a pool of peptides when a peptide pool was used for the stimulations. Once a CTL line was established, it was tested against target cells pulsed with the individual peptides.

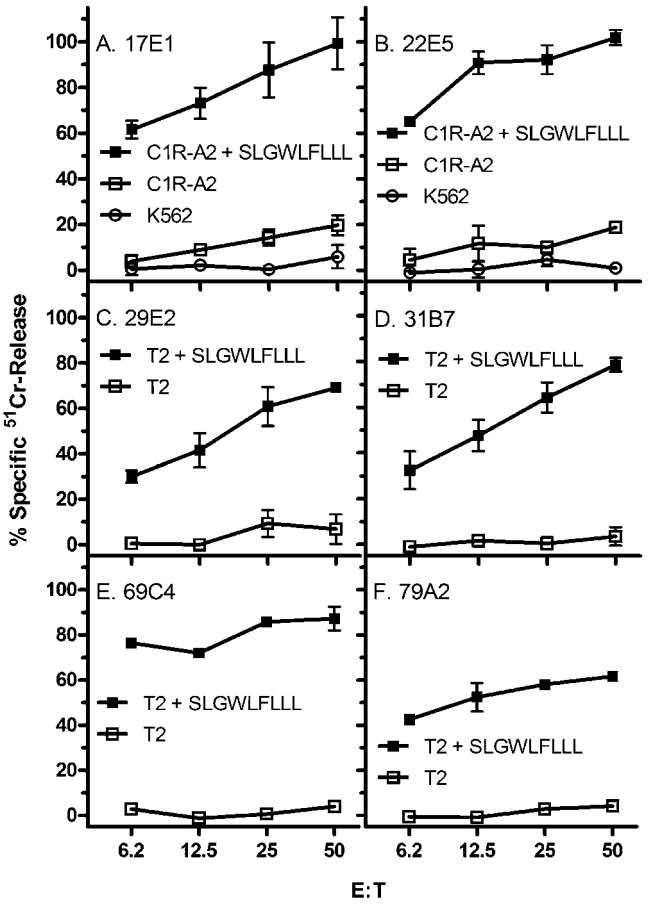

FIGURE 1. Peptide reactivity of anti-CD3 expanded, SLGWLFLLL (SLG) peptide-stimulated microcultures. Expanded microcultures were tested in a ^{51}Cr-release assay against the indicated targets. Six independent cultures are shown, each derived from a separate donor (A-F). C1R-A2 and T2 are HLA-A2^{+} targets.

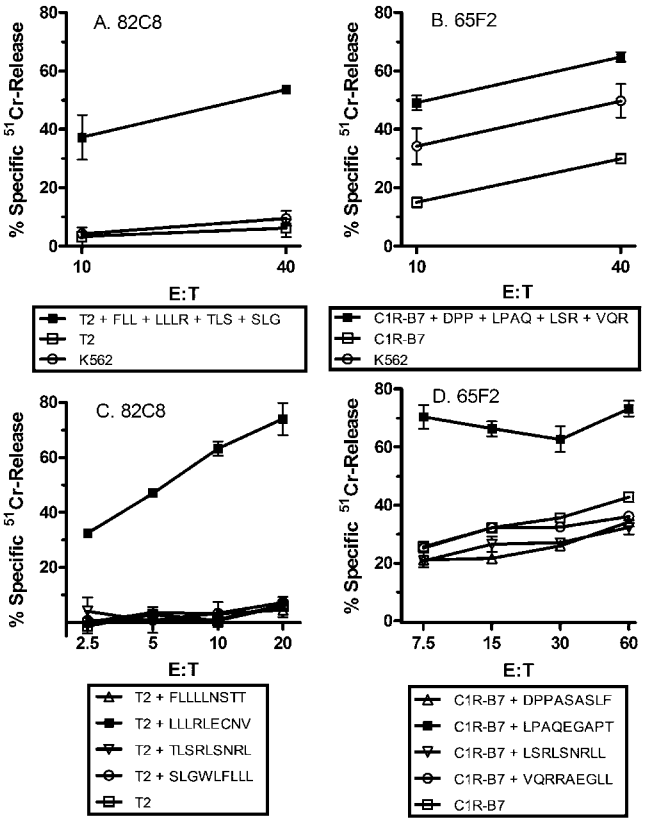

FIGURE 2. Peptide specificity of peptide mix-stimulated microcultures. Microcultures 82C8 (A) and 65F2 (B) were tested in a ^{51}Cr-release assay against targets, expressing either a mix of peptides predicted to associate with HLA-A2 (A) or HLA-B7 (B). After expansion with anti-CD3, 82C8 (C) and 65F2 (D) were tested against the individual peptides, which comprised the original mix. T2 is an HLA-A2^{+} target; C1R-B7 is an HLA-B7^{+} target. Peptides used were FLLLNSTT (FLL), LLLRLECNV (LLLR), TLSRLSNRL (TLS), SLGWLFLLL (SLG), DPPASASLF (DPP), LPAQEGAPT (LPAQ), LSRLSNRLL (LSR), and VQRRAEGLL (VQR).

MHC Restriction of the SLGWLFLLL and LLLRLECNV Peptides

To confirm that the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2, the respective CTL cultures were tested against a panel of target cells that were either matched or unmatched for the class I MHC molecule of interest. Included among these targets were C1R-A2 and C1R-B7 that are class I MHC gene transfectants (HLA-A*0201 and HLA-B*0702, respectively) of the class I MHC null cell line, Hmy2.C1R. These transfectants can be used to unambiguously determine the class I MHC restriction of a CTL line or clone. The target cells were incubated in the presence of the test peptide and then tested for their susceptibility to lysis by the peptide-specific CTL.

CTL lines 69C4 and 22E5, specific for the SLGWLFLLL peptide, recognized the HLA-A2 positive cell lines T2 and C1R-A2 when pulsed with peptide, but did not recognize the HLA-A2 negative MST and C1R-B7 lines when pulsed with peptide (Fig. 3A, B). The same results were obtained with the CTL line 82C8, specific for the LLLRLECNV peptide (Fig. 3C). These results demonstrate that both the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2.

Peptide Dose-response of Peptide-specific CTL

The relative affinity of the peptide-specific CTL lines was determined by testing the ability of the CTL to recognize target cells incubated with 10-fold dilutions of peptide, beginning at 10 μg/mL (~10 μM). CTL lines recognizing the SLGWLFLLL peptide showed a broad range of peptide concentrations over which half-maximal killing was achieved, with most having half-maximal activity between 0.1 and 10 nM (Fig. 4A). CTL lines recognizing the LLLRLECNV peptide had half maximal activity between 1 and 20 nM (Fig. 4B).

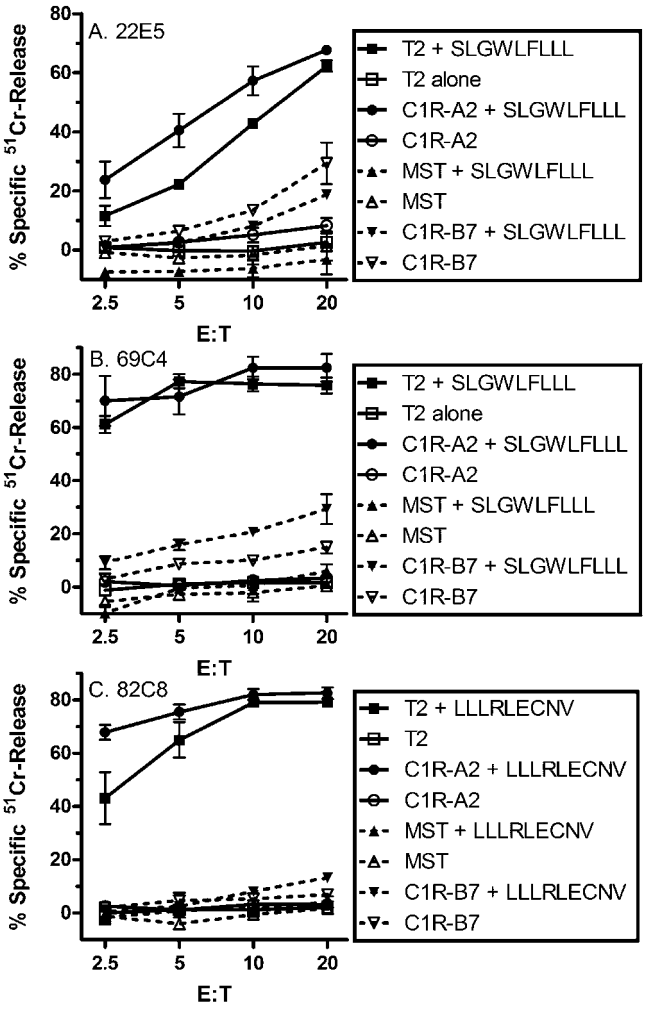

FIGURE 3. MHC restriction of the SLGWLFLLL (SLG) and LLLRLECNV (LLLR) peptides. CTL lines 22E5 (A), 69C4 (B), and 82C8 (C) were tested in a ^{51}Cr-release assay for their ability to kill the indicated targets. Solid symbols indicate that the targets cells were pulsed with 2.5 μg/mL of the indicated peptide as described in the Materials and Methods. Open symbols indicate that the targets were not pulsed with peptide; solid lines, indicate that the targets are HLA-A2$^+$; dashed lines indicate that the targets are HLA-A2$^-$.

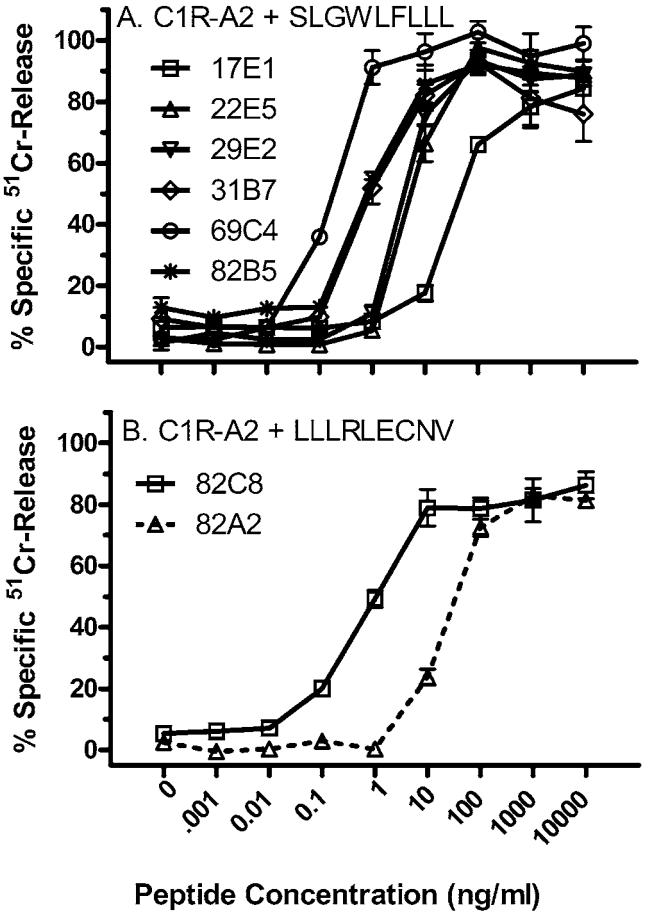

FIGURE 4. Peptide dose-response of peptide-specific CTL. Target cells were created by preincubating C1R-A2 cells for 1 hour at 37°C with the indicated concentrations of the SLGWLFLLL peptide (A) or the LLLRLECNV peptide (B). The targets were then used in a standard ^{51}Cr-release assay with the indicated CTL lines. CTL were used at an E:T of 5:1.

Recognition of Tumors by Peptide-specific CTL

To determine if the peptide-specific CTL also recognize tumor cells, the CTL were tested for their ability to recognize tumors expressing both the appropriate class I MHC molecule and the TAG gene. CTL lines 22E5 and 69C4 (SLGWLFLLL specific) recognized some, but not all, tumors expressing both HLA-A2 and the TAG genes; however, tumors expressing only HLA-A2 or the tumor antigen alone were not recognized (Figs. 5A, B, and data not shown). Likewise, CTL line 82C8 (LLLRLECNV specific) recognized some, but not all tumor lines expressing HLA-A2 and the TAG genes; however, tumors expressing either the HLA-A2 or the tumor antigen alone were not recognized (Fig. 5C and data not shown).

The tumor reactivity of the CTL lines was further confirmed in cold target inhibition experiments. The recognition of ^{51}Cr-labeled DM13 tumor cells (HLA-A2$^+$, TAG$^+$) by the CTL line 69C4 (HLA-A2–restricted, SLGWLFLLL-specific) was inhibited by unlabeled SLGWLFLLL-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Fig. 6A). Similarly, the recognition of ^{51}Cr-labeled DM6 tumor cells (HLA-A2$^+$, TAG$^+$) by the CTL line 82C8 (HLA-A2–restricted, LLLRLECNV-specific) was inhibited by unlabeled LLLRLECNV-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Fig. 6B). These results confirm that the TAG-derived SLGWLFLLL and LLLRLECNV peptides are naturally processed and presented by HLA-A2 on tumor cells.

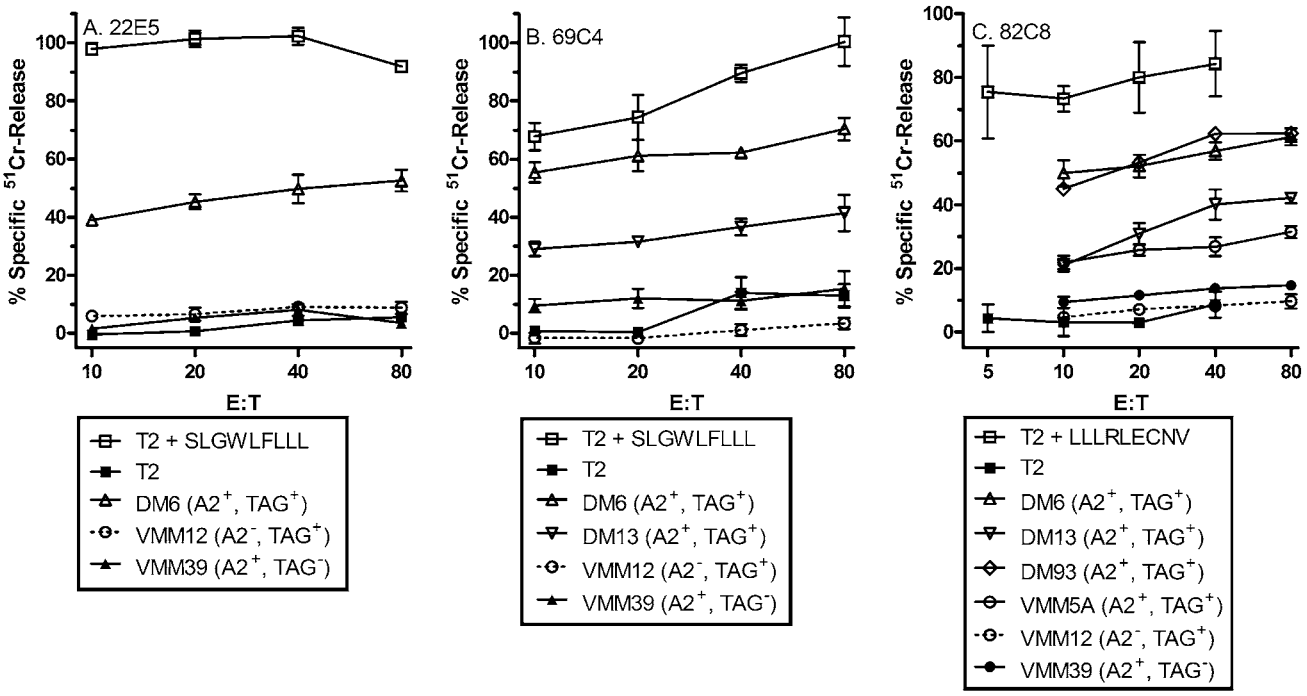

FIGURE 5. Recognition of tumor lines by peptide-specific CTL. CTL lines 22E5 (A), 69C4 (B), and 82C8 (C) were tested in a standard ^{51}Cr-release assay against the indicated targets. Open symbols indicate that the target was incubated with the indicated TAG peptide or expresses the TAG gene; closed symbols indicate that the target was neither incubated with the cognate TAG peptide nor expressed the TAG gene. Solid lines indicate that the target cells are matched with the CTL for expression of HLA-A2; dashed lines indicate that the target cells do not share HLA-A2 in common with the CTL.

DISCUSSION

Cancer/testis antigen expression has most often been studied at the mRNA level by PCR analysis.[30,31] In melanoma, mRNA expression for different cancer/testis antigens ranges from about 4% to 90%.[21,30] TAG (~90%) and MAGE-3 (~50% to 90%) are the most frequently expressed cancer/testis antigens, whereas SAGE is infrequently expressed (4%). Many cancer/testis antigens including BAGE, CT7, GAGE, MAGE-1, NY-ESO-1, and SSX-2 are expressed in about 25% to 75% of the melanoma samples tested. In a study of 8 cancer/testis antigens in 47 different melanoma tumors, 91% expressed at least 1 of the antigens, and 13% expressed all of the antigens.[30] The present work extends our previous findings with TAG in melanoma by demonstrating that the family of genes is expressed in 23% to 59% of 22 fresh melanomas tested (Table 2). Although this level of expression is somewhat less than that found in cultured melanoma cell lines, it is still a relatively high frequency of expression in comparison with other cancer/testis antigens.

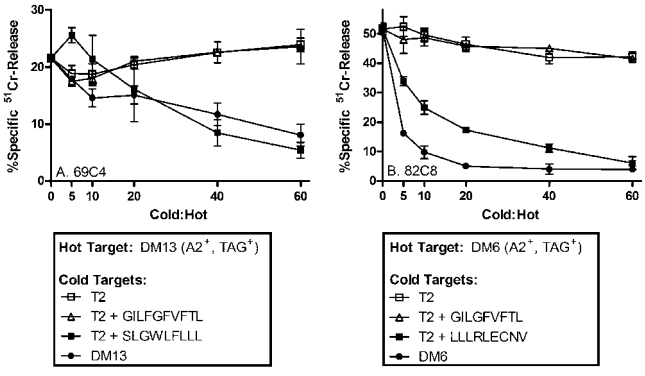

FIGURE 6. Cold target inhibition analysis of peptide-specific CTL. CTL were preincubated for 1 hour at 37°C with the indicated ratio of cold targets, after which the hot targets were added and incubation continued for an additional 4 hours. CTL lines 69C4 and 82C8 were used at an E:T of 40:1.

As cancer/testis antigens are expressed in a wide variety of cancers of diverse histologic types,[18] it was also of interest to determine if tumors of epithelial origin expressed the TAG genes. Established tumor lines including breast, colon, lung, ovarian, pharyngeal, and tongue all expressed TAG, with TAG-1 being the gene most often expressed and at the highest levels (Table 1). A similar finding was also observed with brain tumors which, like melanoma cells, are of an ectodermal lineage (Table 1). As is the case with uncultured melanoma cells, the TAG genes can also be detected in uncultured ovarian tumor cells (Table 2). The expression of the TAG genes in a relatively high frequency of tumors of different tissue origins indicates that TAG-derived antigens would be useful components of vaccines targeting a variety of malignancies.

A frequent goal when immunizing with tumor vaccines is to elicit a tumor-specific CTL response. A

common approach to designing such vaccines is to include 1 or more short antigenic peptides (most often 9 amino acids in length) capable of binding to class I MHC molecules. Class I MHC molecules that are prevalent in the population are most often chosen, because it maximizes the utility of the vaccine at the population level. For example, by concentrating on the 3 most prevalent class I MHC molecules in the white population (ie, HLA-A1 = 28.1%, A2 = 49.1%, and A3 = 25.0%),[32] coverage for approximately 82% of the population can be obtained. By targeting additional prevalent class I MHC molecules (HLA-B7 = 22.9%, B8 = 17.9%), it is possible to approach coverage of the entire population. In the present study, we used 2 predictive approaches to identify candidate TAG derived peptides that could be presented by HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8.[23,24]

A challenge presented by using the predictive approach to antigen identification is that different predictive algorithms result in different peptide rankings. By combining the results of 2 or more predictive algorithms, it is possible to minimize this limitation by focusing on the top-ranked peptides from different algorithms, an approach that has been shown to work with both prostate specific membrane antigen[26] and carcinoembryonic antigen.[27] We have applied that approach to the cancer/testis antigen TAG and have attempted to identify new peptide antigens presented in association with HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 (Table 3). Using this approach, we tested a total of 15 peptides that were predicted to bind to 1 or more of these class I MHC molecules. Of these peptides, 4 were shown to elicit a response. Of these, LSRLSNRLL was initially shown to be recognized, but as the CTL line eventually lost recognition of the peptide, it was not studied further. CTL that recognized LPAQEGAPT in association with HLA-B7 were also generated, but although the CTL could recognize tumor expressing HLA-B7 and the TAG genes, the specificity of the CTL could not be confirmed in a cold target inhibition experiment.

Two antigenic peptides restricted by HLA-A2 were identified. The SLGWLFLLL peptide was recognized by CTL derived from 7 different individuals. This peptide could sensitize targets for lysis with half-maximal killing occurring at 0.1 to 10 nM, which suggests that the CTL have a high affinity for the peptide/HLA-A2 complex and makes it likely that the complex could be recognized on the surface of a tumor cell, with such recognition occurring on DM6 and DM13 melanomas. Not all HLA-A2$^+$, TAG$^+$ tumors were recognized by the CTL, however, indicating that either the peptide/MHC complex is present at very low levels on the surface of some tumor cells or that not all tumors positive for TAG expression by PCR express the TAG protein. To begin to address this question, we have recently produced recombinant TAG-1 and TAG-2 proteins for the purpose of generating TAG-specific antisera.

The LLLRLECNV peptide stimulated an HLA-A2–restricted response from 2 of 6 different donors. The peptide sensitized targets for lysis at 1 to 20 nM, suggesting that these CTL had receptors of a slightly lower affinity than those used to recognize the SLGWLFLLL peptide. In contrast to CTL recognizing the SLGWLFLLL peptide, CTL recognizing the LLLRLECNV peptide recognized multiple tumors expressing both HLA-A2 and TAG. Taken together with the results obtained with the SLGWLFLLL peptide-specific CTL, this suggests that the TAG protein is expressed in many tumors positive for TAG expression by PCR and that the LLLRLECNV epitope is selectively expressed over the SLGWLFLLL epitope.

It is common to use peptide binding experiments as an intermediate step between the predictive step and the CTL elicitation step, with only peptides demonstrating high affinity binding being selected for further study. The experiments of Lu and Celis[26,27] have demonstrated, however, that it is possible to dispense with peptide binding experiments when using a predictive approach to peptide epitope identification. Because binding experiments can be both time-consuming and expensive to perform, their elimination can greatly streamline antigen identification. The results obtained here confirm that peptide antigens can be successfully identified in the absence of performing preliminary binding experiments. Importantly, our results also demonstrate that the antigen identification process can be further consolidated and made more efficient by simultaneously testing multiple peptides. Although there is a theoretical concern that competition among multiple peptides for binding to a limited number of class I MHC molecules might preclude the identification of an antigenic peptide in a peptide mix, we have successfully used the approach here to identify 3 different peptides when 3 or 4 peptides are included in the mix. Because the in vitro stimulations are resource intensive experiments to perform, the ability to simultaneously screen up to 4 peptides will greatly enhance the utility of the predictive approach to peptide epitope antigen identification.

In combination with a previously identified HLA-A3–restricted TAG epitope,[21] a total of 3 TAG-derived epitopes have now been identified from TAG, including 2 HLA-A2–restricted epitopes and 1 HLA-A3–restricted epitope. Because the TAG family of genes is expressed in a high percentage of melanomas and in a variety of tumors of epithelial origin, these epitopes are ideal candidates for inclusion in a vaccine for the therapeutic treatment of a variety of malignancies, for the ex vivo stimulation of T cells for use in adoptive therapy, and as reagents for studying the T-cell–mediated immune response to tumors.

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Cancer Immunol Immunother
DOI 10.1007/s00262-008-0582-6

APPENDIX 3

ORIGINAL ARTICLE

Treatment of ovarian cancer cell lines with 5-aza-2′-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules

Sara J. Adair · Kevin T. Hogan

Received: 26 June 2008 / Accepted: 21 August 2008

Abstract
Purpose To test the hypothesis that decrease in DNA methylation will increase the expression of cancer-testis antigens (CTA) and class I major histocompatibility complex (MHC)-encoded molecules by ovarian cancer cells, and thus increase the ability of these cells to be recognized by antigen-reactive $CD8^+$ T cells.
Methods Human ovarian cancer cell lines were cultured in the presence or absence of varying concentrations of the DNA demethylating agent 5-aza-2′-deoxycytidine (DAC) for 3–7 days. The expression levels of 12 CTA genes were measured using the polymerase chain reaction. The protein expression levels of class I MHC molecules and MAGE-A1 were measured by flow cytometry. T cell reactivity was determined using interferon-γ ELISpot analysis.
Results DAC treatment of ovarian cancer cell lines increased the expression of 11 of 12 CTA genes tested including MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, and TAG-2c. In contrast, DAC treatment decreased the already low expression of the MAGE-A2 gene by ovarian cancer cells, a finding not previously observed in cancers of any histological type. DAC treatment increases the expression of class I MHC molecules by the cells. These effects were time-dependent over a 7-day interval, and were dose-dependent up to 1–3 μM for CTA and up to 10 μM for class I MHC molecules. Each cell line tested had a unique pattern of gene upregulation after exposure to DAC. The enhanced expression levels increased the recognition of 2 of 3 antigens recognized by antigen-reactive $CD8^+$ T cells.
Conclusions These results demonstrate the potential utility of combining DAC therapy with vaccine therapy in an attempt to induce the expression of antigens targeted by the vaccine, but they also demonstrate that care must be taken to target inducible antigens.

Keywords Ovarian cancer · Class I MHC molecules · Cancer-testis antigens · DNA methylation · 5-Aza-2′-deoxycytidine

Abbreviations

CTA	Cancer-testis antigen
CTL	Cytotoxic T lymphocyte
DAC	5-Aza-2′-deoxycytidine
DNMT	DNA methyltransferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction

Introduction

Surgery and chemotherapy are the mainstays of ovarian cancer therapy but their use only results in a 5-year survival rate of less than 45% [44]. An additional treatment approach under investigation involves the immunization of patients with cancer antigen-derived peptides with the goal being to stimulate antigen-specific cytotoxic T lymphocytes (CTL) that will recognize and kill the tumor [25, 41, 42].

Electronic supplementary material The online version of this article (doi:10.1007/s00262-008-0582-6) contains supplementary material, which is available to authorized users.

S. J. Adair · K. T. Hogan (✉)
Department of Surgery and the Human Immune Therapy Center, University of Virginia, Box 801359, Charlottesville, VA 22908, USA
e-mail: kh6s@virginia.edu

Cancer-testis antigens (CTA) represent a promising group of antigens whose inclusion in an ovarian cancer vaccine may prove efficacious. CTAs have been defined on the basis of their expression in a variable proportion of tumors of many different histological types, expression in the testis, and limited or no expression in other normal tissue [5, 31, 46, 61]. A potential drawback to the use of CTAs in vaccines is that CTAs are expressed in only a subset of all tumor samples studied, with ovarian cancer being considered a moderate expressor in comparison to other cancers [4, 46]. Additionally, CTA expression is frequently heterogeneous within a tumor line or sample [15, 26–28]. Because of these limitations, some ovarian cancer cells may escape immune recognition even if a vaccine based on CTAs is capable of inducing a strong, therapeutic CTL response. The ability to induce high levels of CTA expression in all ovarian cancer cells would overcome these limitations.

Treatment of melanoma cell lines with the demethylating agent 5-aza-2′-deoxycytidine (DAC) was first shown to upregulate the expression of the CTA MAGE-A1 [56]. This observation has since been extended to additional CTAs including BAGE [34], CAGE [7], GAGE1-2 [34, 51], GAGE-1-6 [8, 34, 51], MAGE-A2 [8, 51], MAGE-A3 [8, 21, 34, 51, 58], MAGE-A4 [8, 51], MAGE-A10, and NY-ESO-1 [8, 51, 57]. The enhancement of CTA expression by DNA demethylating agents has also been observed in cells derived from breast cancer [21], colon cancer [30], esophageal cancer [58], gastric cancer [7, 34], lung cancer [58], mesothelioma [51], renal cell cancer [8], and thoracic cancer [57]. Only a single such study has been conducted with ovarian cancer in which microarray analysis demonstrated that DAC treatment of the OVCAR-3 cell line upregulated a variety of CTAs including: CTAG-1A and -1B; MAGE-A1, -A3, -A4, -A6, -A11, -A12, and -B2; and SPAN-X [37]. Upregulation of MAGE-A3 in DAC-treated OVCAR3 was further confirmed by PCR analysis [37]. Absent or low expression of CTAs is associated with promoter region hypermethylation, while high or upregulated expression of CTAs is associated with promoter region hypomethylation [7, 12, 13, 30, 51, 52]. The functional significance of the upregulation of NY-ESO-1 has been demonstrated by the increased recognition of the DAC-treated cells by antigen-specific CTL [8, 57].

The treatment of melanoma [10, 19, 20, 49] and esophageal squamous cell cancers [39] with DAC has also been shown to upregulate the expression of class I major histocompatibility complex (MHC)-encoded molecules. Like the expression of CTAs, the upregulation occurs through the demethylation of the class I MHC gene promoter regions [39]. Promoter methylation primarily regulates class I MHC expression between low and high levels [10, 19, 20], and with the exception of a single cell line [49], generally does not appear to be responsible for the lack of expression of class I MHC molecules [20]. The increased level of class I MHC expression on melanoma cells was functionally significant as it increased the recognition of the cells by antigen-specific CTL [19].

DAC also upregulates the expression of CTAs and class I MHC molecules in vivo. In a xenograft model, BALB/c *nu/nu* mice were engrafted with human melanoma cells and then treated with DAC [9]. After 1 week, the tumors had either de novo or upregulated expression of ten different CTAs tested, as well as upregulated expression of the class I MHC molecules HLA-A1 and HLA-A2. DAC treatment also induced expression of the murine CTA P1A in tumors of five different histological origins when grown in syngeneic mice [22]. It was further shown that the induced P1A was processed and presented by H-2L^{d}, and recognized by P1A-specific, H-2L^{d}-restricted CTL. Importantly, lung metastases were most effectively controlled when tumor bearing mice were pre-treated with DAC and then treated with the adoptive transfer of antigen-specific CTL, while the adoptive transfer of the CTL in the absence of DAC treatment was ineffective.

The effect of DAC on CTA and class I MHC molecule expression has also been assessed in cancer patients. When myelodysplastic syndrome or acute myeloid leukemia patients were treated with DAC over 3 days, MAGE-A1, SSX, and NY-ESO-1 were expressed de novo between 15 and 30 days following treatment [50]. Likewise, when lung, esophageal, and pleural mesothelioma patients were treated with DAC for 3 days and tumor biopsies obtained 1-day following treatment, a third of the samples demonstrated upregulation of NY-ESO-1 or MAGE-A3 [47].

Although CTAs represent a potentially rich source of antigens for inclusion in vaccines designed to treat ovarian cancer, the utility of the individual antigens is limited by their lack of expression in all ovarian cancers [4, 46]. Likewise, targeting of vaccine-induced CTL to ovarian cancer requires that the tumor cells express class I MHC molecules, but the tumors frequently have low levels of class I MHC expression [4]. To overcome these limitations, we sought to determine if the treatment of ovarian cancer cells with DAC would induce or upregulate both CTAs and class I MHC molecule expression.

Materials and methods

Cell culture medium

RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin was further supplemented with 5% fetal bovine serum (RPMI-5FBS),

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10% FBS (RPMI-10FBS), or 10% human AB-serum (RPMI-10HS).

Cell lines

The ovarian cancer lines CAOV-3 [3], CAOV-4 [29], COV413 [32], ES-2 [33], OV-90 [43], OVCAR-3 [23], SK-OV-3 [17], SW626 [18], TOV-21G [43], TOV-112D [43], and TTB-6 [4] were maintained in RPMI-10FBS. C1R-A2 and C1R-A3 were maintained in RPMI-5FBS supplemented with 300 μg/ml G418.

DAC treatment

Cells were counted and plated in new T75 flasks 1-day prior to the treatment. On the day of treatment the media was removed and replaced with media containing the appropriate concentration of DAC (Sigma-Aldrich, St. Louis, MO) that had been freshly dissolved at 1 mM in Dulbecco's phosphate-buffered saline. After incubation with DAC for 3–7 days, the cells were harvested, counted, and then prepared for RNA extraction or flow cytometry.

Flow cytometry

Monoclonal antibodies (mAb) CR11-351 (anti-HLA-A2,A68,A69; γ_1) [45], GAP-A3 (anti-HLA-A3; γ_{2a}) [1], ME1-1.2 (anti-HLA-B7,B27; γ_1) [16], and W6/32 (anti-HLA-A, B, C; γ_{2a}) [40] were produced in our laboratory from the corresponding hybridoma and were used at a final concentration of 10 μg/ml. mAb 3F257 (anti-MAGE-A1; γ_{2a}) (United States Biological, Swampscott, MA) was used at a final concentration of 10 μg/ml.

Class I MHC-specific mAbs were added to 2×10^5 cells and incubated for 60 min on ice. The cells were washed twice, 50 μl of a 1:50 dilution of sheep anti-mouse IgG-FITC (ICN, Irvine, CA) was then added, and the cells were incubated an additional 60 min on ice. The cells were then washed once, fixed with 0.5% paraformaldehyde in PBS, and analyzed on a FACSCalibur instrument (BD Biosciences, San Jose, CA). Binding of the 3F257 antibody was evaluated on cells that had been fixed and permeabilized according to the Cytofix/Cytoperm kit instructions (BD Biosciences). Both the primary and secondary antibodies were diluted in perm/wash buffer.

RNA and cDNA preparation

Total RNA was prepared from 2 to 5×10^6 cells using the RNeasy Mini kit (Qiagen) as per the kit instructions. RNA was quantified by absorbance at 260 nm. Total RNA was converted to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA).

Polymerase chain reaction (PCR)

Primer sequences were as previously published for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [36], MAGE-A1 [2], MAGE-A2 [14], MAGE-A3 [11], MAGE-A4 [11], MAGE-A6 [11], MAGE-A10 [11], MAGE-A12 [11], NY-ESO-1 [55], TAG-1 [24], TAG-2a [24], TAG-2b [24], and TAG-2c [24]. Each of the primer sequences, annealing temperatures, and predicted product sizes has previously been summarized [4].

PCR was performed on 250 ng of cDNA using Platinum *Taq* polymerase (Invitrogen). The PCR mixes were heated to 94°C for 2 min, 30 and 40 cycles of amplification were performed (1 cycle = 30 s denaturation at 94°C, 30 s annealing at the temperature given in Ref. [4], 60 s extension at 72°C), and a final extension completed at 72°C for 5 min. Relative expression levels were determined by visualizing DNA bands on ethidium bromide-stained 1.5% agarose gels and are reported on a scale of 0–8. Level 0 represents the inability to visualize a band at 40 cycles of PCR. Levels 1–4 correspond to progressively brighter bands at 40 cycles of PCR analysis, with 1 being weakly visible and 4 being easily visible. Levels 5–8 correspond to progressively brighter bands at 30 cycles of PCR analysis, with 5 being weakly visible and 8 being easily visible.

ELISpot analysis

$CD8^+$ T lymphocyte lines were generated from patients vaccinated with a mixture of peptides as described [6, 53, 60]. Those selected for use in this study were specific for: (a) the HLA-A2-restricted, MAGE-A10-derived peptide GLYDGMEHL (GLY), (b) the HLA-A3-restricted, MAGE-A1-derived peptide SLFRAVITK (SLF), and (c) the HLA-A3-restricted, NY-ESO-1-derived peptide ASGPGGGAPR (ASG). The peptide SLYNTVATL (GAG) derived from the gag protein of HIV-1 was used as an irrelevant peptide control [6].

Lymphocytes were stimulated and tested by ELISpot analyses as previously described [6]. Briefly, the lymphocytes were incubated with 40 μg/ml peptide for 2 h, washed, resuspended in RPMI-10HS containing 20 U/ml IL-2, and then incubated for 14 days. The cells were then plated in quadruplicate at 25,000 and 75,000 cells per well of interferon-γ coated $\text{MultiScreen}_{\text{HTS}}$ IP plates (Millipore, Billerica, MA). An equal number of peptide-pulsed antigen presenting cells were added to each well and the plates were incubated for 18–20 h. The plates were then washed and biotin-labeled interferon-γ added to each well. Spots were developed using streptavidin conjugated alkaline phosphatase (BD Biosciences, San Jose, CA) and 4-chloro-3-indolyl phosphatesubstrate-Toluidine salt (Pierce, Rock-

ford, IL). Spots were counted using an automated plate reader (Bioreader 4000, Biosys, The Colony, TX).

Human subjects research approval

This research was approved by the University of Virginia Human Investigation Committee in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Results

Effect of the treatment of ovarian cancer cell lines with 1 μM DAC for 3 days on CTA gene expression and class I MHC protein expression

Based on the fact that most studies designed to investigate the effect of DAC treatment on the expression of CTA genes and class I MHC proteins have treated cells for 2–4 days with 1–2 μM DAC [10, 12, 34, 56, 59], we treated 11 ovarian cancer cell lines with 1 μM DAC for 3 days. CTA gene expression was assessed by performing 30 and 40 cycles of PCR analysis. PCR with primers for the housekeeping gene GAPDH was first used to ensure that amplifiable cDNA had been obtained from each cell line. The results of this analysis demonstrated that all of the ovarian cancer cell lines had comparable levels of GAPDH gene expression (data not shown). PCR amplifications were then performed for 12 CTA genes (Table 1, Supplementary Fig. 1). DAC treatment resulted in higher levels of expression of at least 1 CTA gene in ten of the 11 cell lines. Most cell lines had increased levels of expression of 1–5 CTA genes, while one cell line had increased levels of expression of seven CTA genes. The expression levels of the MAGE-A1 and NY-ESO-1 genes were most frequently enhanced while the MAGE-A2, MAGE-A6, and MAGE-A12 genes were not enhanced in any of the cell lines tested. The expression of the remaining CTA genes was shown to increase in 1–3 cell lines each.

Class I MHC protein expression in the untreated and DAC-treated cells was assessed by flow cytometry using mAb W6/32, which recognizes an epitope common to all class I MHC molecules, and mAbs to the individual class I MHC molecules HLA-A2 (mAb CR11-351), HLA-A3 (mAb GAP-A3), and HLA-B7 (mAb ME1-1.2). The treatment resulted in a reproducible, but small increase in HLA-A2 and total class I MHC expression in OV-90 (Supplementary Fig. 2). Conversely, the treatment of SK-OV-3 with DAC did not reproducibly increase HLA-A2, HLA-A3, or total class I MHC expression (Supplementary Fig. 2). The results obtained with OV-90 and SK-OV-3 were representative of those obtained with the

Table 1 The effect of treating ovarian cancer cell lines with DAC on CTA gene expression

DAC treatment	No. of cell lines with an increase/No. of cell lines where an increase could be measured											
Conditions	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A10	MAGE-A12	NY-ESO-1	TAG-1	TAG-2a	TAG-2b	TAG-2C
1 μM, 3 day[a]	8/11	0/11	1/9	2/11	0/11	1/10	0/9	9/11	2/10	3/11	2/11	3/11
10 μM, 7 day[b]	9/9	0/11	9/9	11/11	7/11	9/9	8/9	10/10	8/9	5/9	8/11	4/11
DAC treatment	No. of CTA with an increase/No. of CTA where an increase could be measured											
Conditions	CAOV-3	CAOV-4	COV413	ES-2	OV-90	OVCAR-3	SK-OV-3	SW626	TOV-21G	TOV-112D	TTB-6	
1 μM, 3 day[a]	2/12	3/12	7/12	5/10	0/10	3/10	1/12	2/12	2/12	5/12	1/12	
10 μM, 7 day[b]	9/12	11/12	10/12	7/9	1/7	6/9	10/12	9/12	10/12	9/10	6/10	

Eleven ovarian cancer cell lines were incubated in the presence or absence of DAC under the indicated treatment conditions. The expression levels of the 12 indicated CTA genes were determined by PCR as indicated in the "Materials and methods"

[a] Two independent experiments were performed for this treatment condition. An increase in expression following DAC treatment was defined as an increase of two or more units on the relative expression scale in each of the two experiments. When a particular CTA/cell line pair could not show an increase because the untreated cells in at least one experiment had a relative expression level of 7 or 8 on a scale of 8, these data points were excluded from the denominator

[b] Three independent experiments were performed for this treatment condition. An increase in expression following DAC treatment was defined as an increase in expression in each individual experiment and an increase of two or more units on the relative expression scale in at least two of the experiments. When a particular CTA/cell line pair could not show an increase because the untreated cells in at least one experiment had a relative expression level of 7 or 8 on a scale of 8, these data points were excluded from the denominator

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remaining nine ovarian cancer cell lines (data not shown).

Effect of varying the length of treatment time of ovarian cancer cell lines with 1 μM DAC on CTA gene expression and class I MHC protein expression

The effect of varying the length of treatment time with DAC was studied by comparing CTA gene and class I MHC protein expression levels in selected cell lines (ES-2, OV-90, OVCAR-3, and SK-OV-3) treated with 1 μM DAC for 3, 5, and 7 days. GAPDH gene expression in the four cell lines was not affected by any length of treatment (Fig. 1). MAGE-A1 gene expression was highest at day 7 for three of the four cell lines, and NY-ESO-1 gene expression was highest at day seven for all four cell lines. Similar results were observed when class I MHC molecule expression was measured by flow cytometry (Fig. 2 and data not shown). The highest levels of expression of HLA-A2, HLA-B7, and overall class I MHC molecule expression on OVCAR-3 were observed on day 7 following treatment. For SK-OV-3, expression of HLA-A2, HLA-A3, and overall class I MHC expression was increased to similar levels following 5 and 7 days of treatment and in all cases exceeded that observed following 3 days of treatment. Consequently, subsequent experiments were conducted with 7 days of DAC treatment.

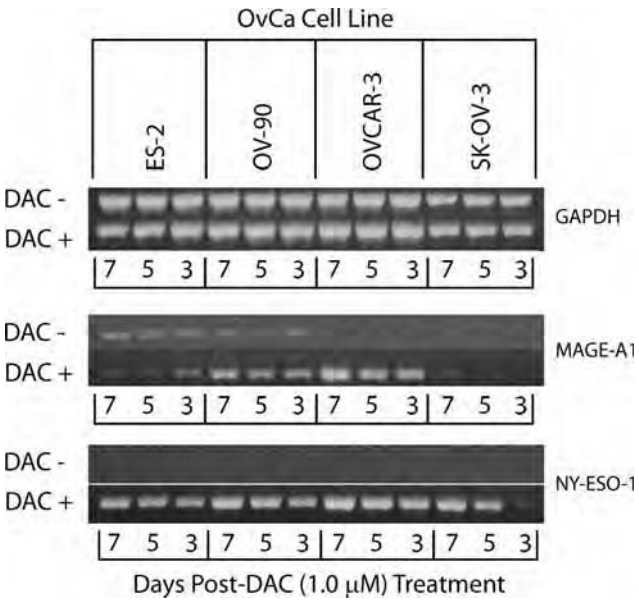

Fig. 1 Time course analysis of the effect of treating ovarian cancer cell lines with 1 μM DAC for 3, 5, or 7 days on CTA gene expression. The indicated ovarian cancer cell lines were incubated for 3, 5, or 7 days in the presence or absence of 1 μM DAC, harvested, and cDNA prepared. CTA gene expression was determined by PCR as indicated in the "Materials and methods". *Bands* were visualized on ethidium bromide stained 1.5% agarose gels. The results from one of two to three independent experiments are shown

Effect of treating ovarian cancer cell lines with varying concentrations of DAC on CTA gene expression and class I MHC protein expression

The optimal concentration of DAC needed to enhance the expression of CTA genes and class I MHC proteins was next determined. Ovarian cancer cell lines were untreated, or treated with varying concentrations of DAC (0.1–30 μM) for 7 days. PCR analysis of OVCAR-3 and SK-OV-3 showed that the treatment did not affect GAPDH gene expression (Fig. 3). Conversely, the expression of CTA genes was enhanced by increasing concentrations of DAC, with maximal expression requiring a minimal concentration of 1–3 μM. Maximal expression of class I MHC molecules required a higher concentration of DAC, with 10 μM appearing to be optimal (Fig. 4). Based on the results of dose response and time course experiments, 7 days of incubation with 10 μM DAC was adopted as the standard treatment to enhance CTA gene expression and class I MHC protein expression.

Effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on CTA gene expression, MAGE-A1 protein expression, and class I MHC protein expression

Having determined the optimal concentration and incubation period for DAC treatment, we next treated 11 ovarian cancer cell lines with 10 μM DAC for 7 days. CTA gene expression was determined with both 30 and 40 cycles of PCR analysis. Results of a single experiment analyzed with 30 cycles of PCR demonstrate several points (Fig. 5). First, CTA genes are not readily detectable in most untreated cell lines when tested with 30 cycles of PCR amplification. ES-2 and OV-90 are exceptions in that many of the CTA genes can be detected in these cell lines without prior exposure to DAC. Second, treatment with DAC can be very effective in upregulating the expression of some CTA genes (MAGE-A1, MAGE-A3, MAGE-A10, NY-ESO-1) while it has little (MAGE-A4) to no (MAGE-A2) effect on the expression of other CTA genes when measured at 30 cycles of PCR. Third, the increased expression of a particular CTA gene in one cell line does not predict that it will be increased in all cell lines (MAGE-A6, NY-ESO-1). Fourth, the increased expression of a particular CTA gene in one cell line is not predictive of other CTA genes being increased in the same cell line.

The sensitivity of this analysis was increased by also conducting the CTA gene expression measurements with 40 cycles of PCR amplification. The combined results of 30 and 40 cycles of PCR analysis are presented in Table 1 and Supplementary Fig. 3. The number of CTA genes with enhanced expression ranged from 1 to 11 per cell line, with most cell lines having enhanced expression of 6 to 11 CTA genes. Correspondingly, the expression of nine of the 12

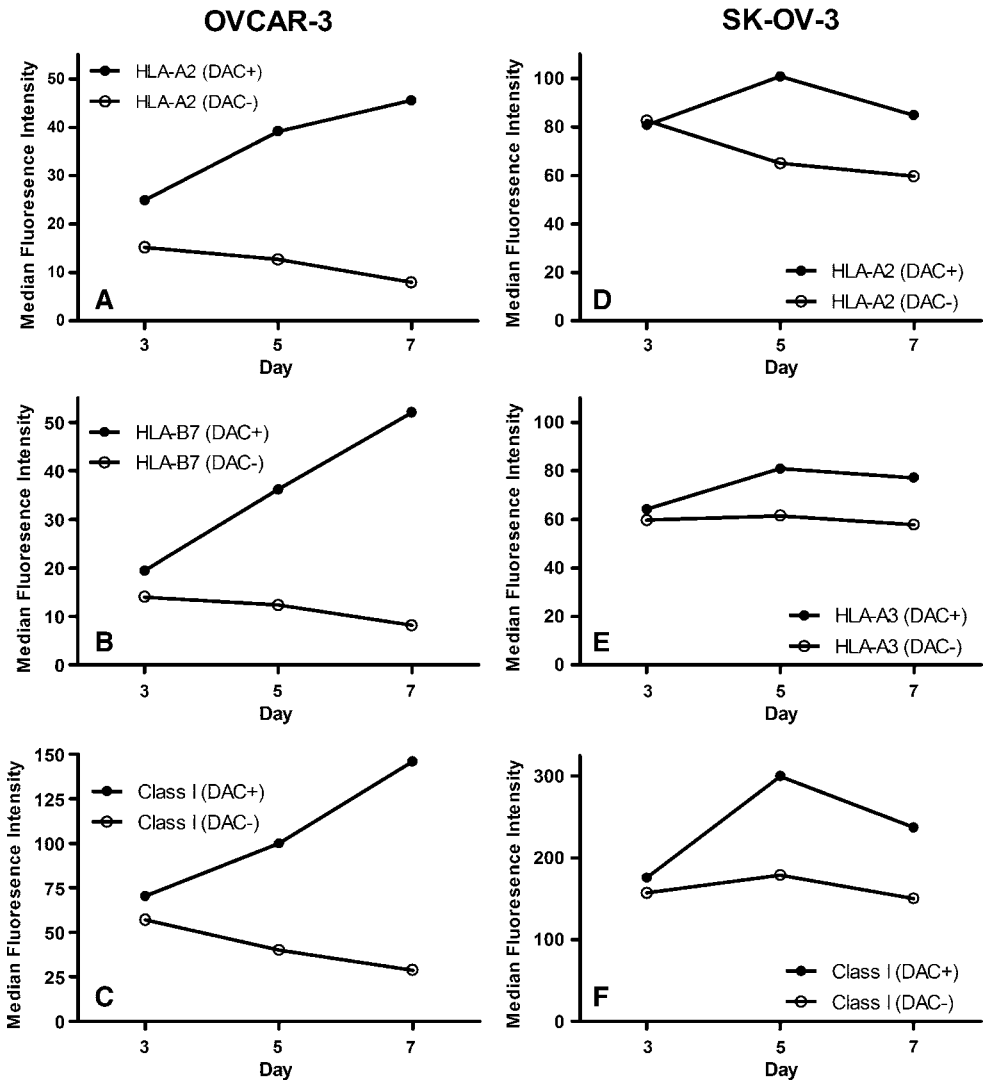

Fig. 2 Time course analysis of the effect of treating ovarian cancer cell lines with 1 μM DAC for 3, 5, or 7 days on class I MHC protein expression. The indicated ovarian cancer cell lines were incubated for 3, 5, or 7 days in the presence or absence of 1 μM DAC and were then harvested. Class I MHC protein expression levels were determined by flow cytometry using mAb CR11-351 to detect HLA-A2 (**a**, **d**), mAb GAP-A3 to detect HLA-A3 (**e**), mAb ME1-1.2 to detect HLA-B7 (**b**), and mAb W6/32 (**c**, **f**) to detect all class I MHC molecules. The results from one of three independent experiments are shown

CTA genes was enhanced in at least seven different ovarian cell lines. Expression of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, and NY-ESO-1 was increased in every evaluable cell line. Only MAGE-A2 did not increase in any cell line, and in fact, tended to decrease from low to no expression following treatment (Supplementary Fig. 3).

The expression of the MAGE-A1 protein was measured to determine if increased CTA gene expression levels also lead to an increase in the expression of the corresponding protein. Although many lines showed a large increase in the expression of the MAGE-A1 gene when treated with 10 μM DAC for 7 days (Table 1, Supplementary Fig. 3), the cell lines did not show a correspondingly large increase in protein expression (Fig. 6). MAGE-A1 protein expression increased greater than threefold in CAOV-3 and TOV-21G, and greater than 1.9-fold in OV-90, OVCAR-3, and TOV-112D.

Class I MHC protein expression was also assessed following treatment of the 11 ovarian cancer cell lines with 10 μM DAC for 7 days (Fig. 7). Overall, two patterns of expression emerged. When class I MHC molecules are already expressed at high levels (ex. CAOV-3 and CAOV-4) there was no to only a modest enhancement of expression. When protein expression was low, increases in expression levels ranged from about two- to tenfold; however, despite the high relative increase in expression, the overall protein expression levels remained below that found in a cell lines naturally expressing high levels of class I MHC molecules.

Recognition of DAC-treated (10 μM for 7 days) ovarian cancer cells by antigen-reactive $CD8^+$ T lymphocytes

To determine if enhanced expression of CTA and class I MHC molecules leads to increased recognition of the treated cells by T lymphocytes, antigen-reactive $CD8^+$ T lymphocytes that recognize peptide antigens derived from the MAGE-A1, MAGE-A10, and NY-ESO-1 proteins were

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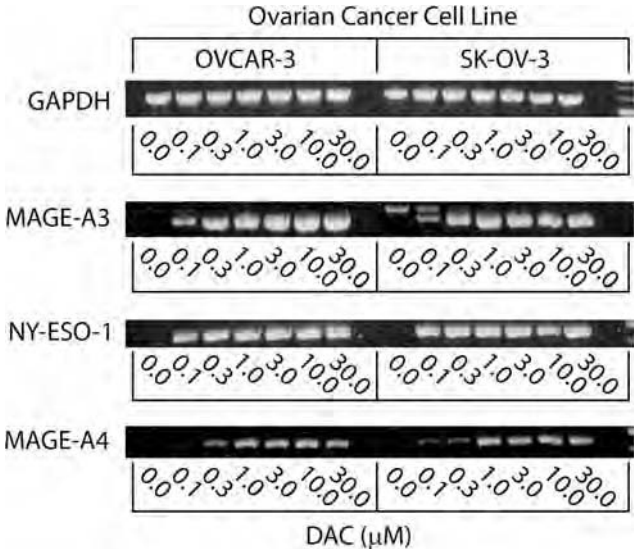

Fig. 3 Dose response analysis of the effect of treating ovarian cancer cell lines with 0.1–30 μM DAC for 7 days on CTA gene expression. The indicated ovarian cancer cell lines were incubated for 7 days in the presence or absence of 0.1–30 μM DAC, harvested, and cDNA prepared. CTA gene expression was determined by PCR as indicated in the "Materials and methods". *Bands* were visualized on ethidium bromide stained 1.5% agarose gels. The results from one of two independent experiments are shown

tested in an interferon-γ ELISpot assay for their ability to recognize DAC-treated and untreated cells. $CD8^+$ T lymphocytes that recognize the NY-ESO-1-derived peptide ASG in association with HLA-A3 (Fig. 8a, c) were tested against SK-OV-3, SW626, and TOV-112D (all NY-ESO-1^+, HLA-$A3^+$) as potential positive stimulator cells and COV413 (NY-ESO-1^+, HLA-$A3^-$) as a control cell line (Fig. 8b, d). DAC treatment of SW626, but not the remaining cell lines, led to an increase in T cell responses to those tumor cells. $CD8^+$ T lymphocytes that recognize the MAGE-A1-derived peptide SLF in association with HLA-A3 (Fig. 8e, g) were tested against the same cell lines, all of which also express MAGE-$A1^+$ (Fig. 8f, h). Treatment of SW626 with DAC was again shown to lead to an increase in the ability of the cell line to trigger interferon-γ release by the antigen-specific $CD8^+$ T lymphocytes. Finally, $CD8^+$ T lymphocytes that recognize the MAGE-A10-derived peptide GLY in association with HLA-A2 (Fig. 8i) were tested for interferon-γ release against CAOV-4, COV413, and OVCAR-3 (all MAGE-$A10^+$, HLA-$A2^+$) as potential stimulators and TOV-112D (MAGE-$A10^+$, HLA-$A2^-$) as a control cell line (Fig. 8j). Treatment of these cell lines with DAC did not significantly change their ability to stimulate antigen-specific $CD8^+$ T lymphocytes.

Discussion

The results presented here clearly indicate that the treatment of ovarian cancer cells with DAC can lead to an enhancement in expression of CTA genes (Table 1, Supplementary Fig. 3). In comparison to other studies in which 3–4 days of treatment with 1–2 μM DAC was routinely used to

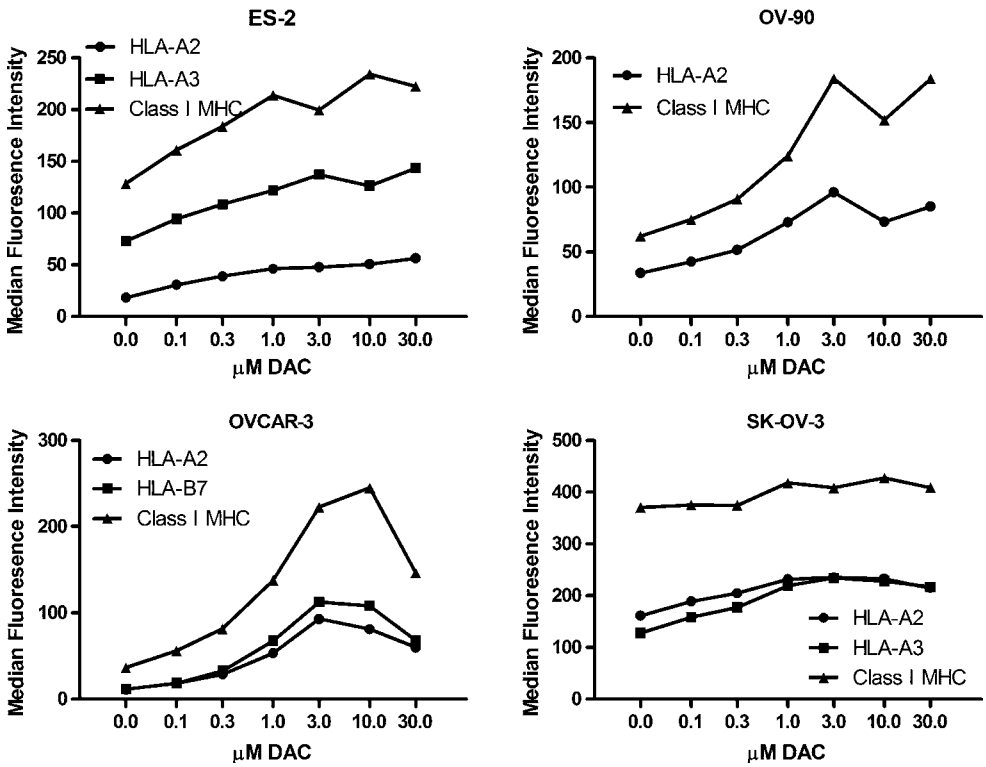

Fig. 4 Dose response analysis of the effect of treating ovarian cancer cell lines with 0.1–30 μM DAC for 7 days on class I MHC protein expression. The indicated ovarian cancer cell lines were incubated for 7 days in the presence or absence of 0.1–30 μM DAC and were then harvested. Class I MHC protein expression levels were determined by flow cytometry using mAb CR11-351 to detect HLA-A2, mAb GAP-A3 to detect HLA-A3, mAb ME1-1.2 to detect HLA-B7, and mAb W6/32 to detect all class I MHC molecules. The results from one of two independent experiments are shown

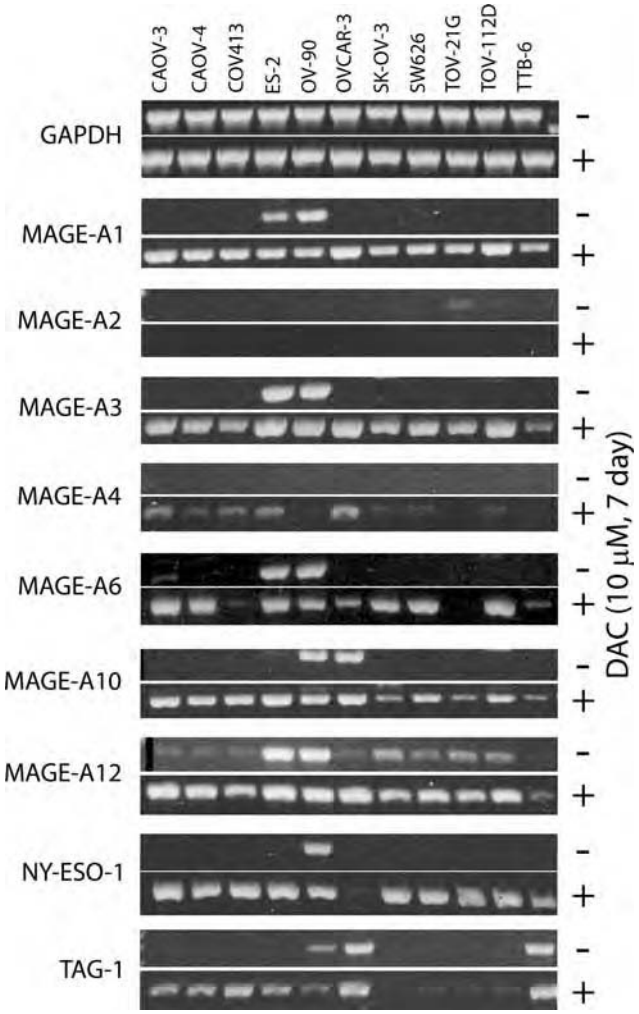

Fig. 5 The effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on CTA gene expression. Eleven ovarian cancer cell lines were incubated for 7 days in the presence (+) or absence (−) of 10 μM DAC, harvested, and cDNA prepared. CTA-specific PCR was then performed and the amplification products visualized on ethidium bromide stained 1.5% agarose gels. The 30-cycle results from one of three independent experiments are shown

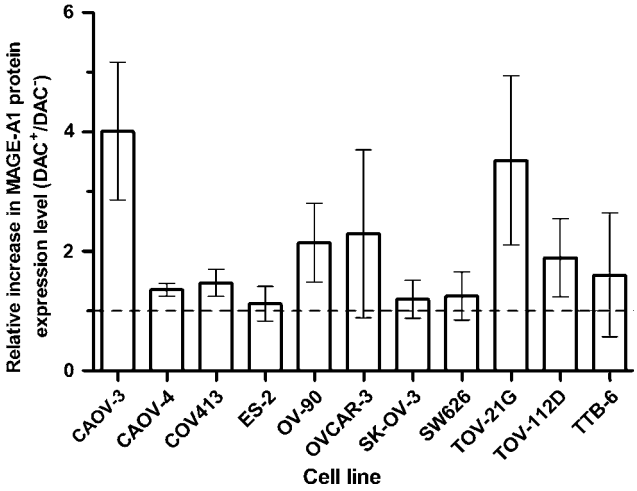

Fig. 6 The effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on MAGE-A1 protein expression. Eleven ovarian cancer cell lines were incubated for 7 days in the presence or absence of 10 μM DAC and were then harvested. The cells were fixed and permeabilized and then MAGE-A1 expression levels determined by flow cytometry using mAb 3F257. The data are presented as mean ± SEM of two independent experiments and are the ratio of the fluorescence intensity of DAC-treated cells to the fluorescence intensity of the untreated cells. The *dashed line* represents a 1:1 ratio of the fluorescence intensities

upregulate CTA gene expression [10, 12, 34, 56, 59], ovarian cancer cell lines required 7 days of treatment with DAC at 1–3 μM to achieve the highest levels of CTA gene expression (Figs. 1, 3). The need for a lengthier treatment time may reflect the longer doubling times that ovarian cell lines have in comparison to melanoma cell lines (unpublished observations). Because DAC acts by inhibiting methylation of a newly synthesized DNA strand, it would be expected that the rate at which demethylation will occur would be a function of the replicative rate of the cells.

It is important to note, however, that DAC treatment is not an universal panacea for upregulating CTA gene expression. MAGE-A2 gene expression, for example, does not increase, but rather decreases in expression following DAC treatment (Supplementary Fig. 3). This result stands in contrast to the results obtained with CTA genes such as MAGE-A1, MAGE-A3, MAGE-A10, and NY-ESO-1, which when ordinarily expressed at low levels undergo a dramatic upregulation following DAC treatment. It is also evident that there is heterogeneity in the response to DAC treatment, both with respect to how frequently the expression of a particular CTA gene is upregulated in different cell lines, and with respect to how many CTA genes are upregulated in a particular cell line. Such heterogeneous response patterns of CTA gene expression are not unique to ovarian cancer cell lines, but have been demonstrated in other cancers [8, 12, 51, 52]. These findings likely reflect the multiple control mechanisms that can affect the expression of any particular gene, as well as the fact that promoter demethylation will have little functional consequence if the gene in question is no longer functional due to a genetic defect.

It is not increased CTA gene expression per se that leads to increased recognition of tumor cells by CTA-specific T cells, but rather it is increased peptide production which can occur through a combination of increased protein production and/or increased protein degradation. There are few CTA-specific antibodies available to measure protein expression levels, although a number of antibodies have been developed which recognize MAGE-A proteins. Although most of the antibodies with specificity for MAGE-A proteins recognize multiple members of the MAGE-A family, the mAb 3F257 is MAGE-A1 specific

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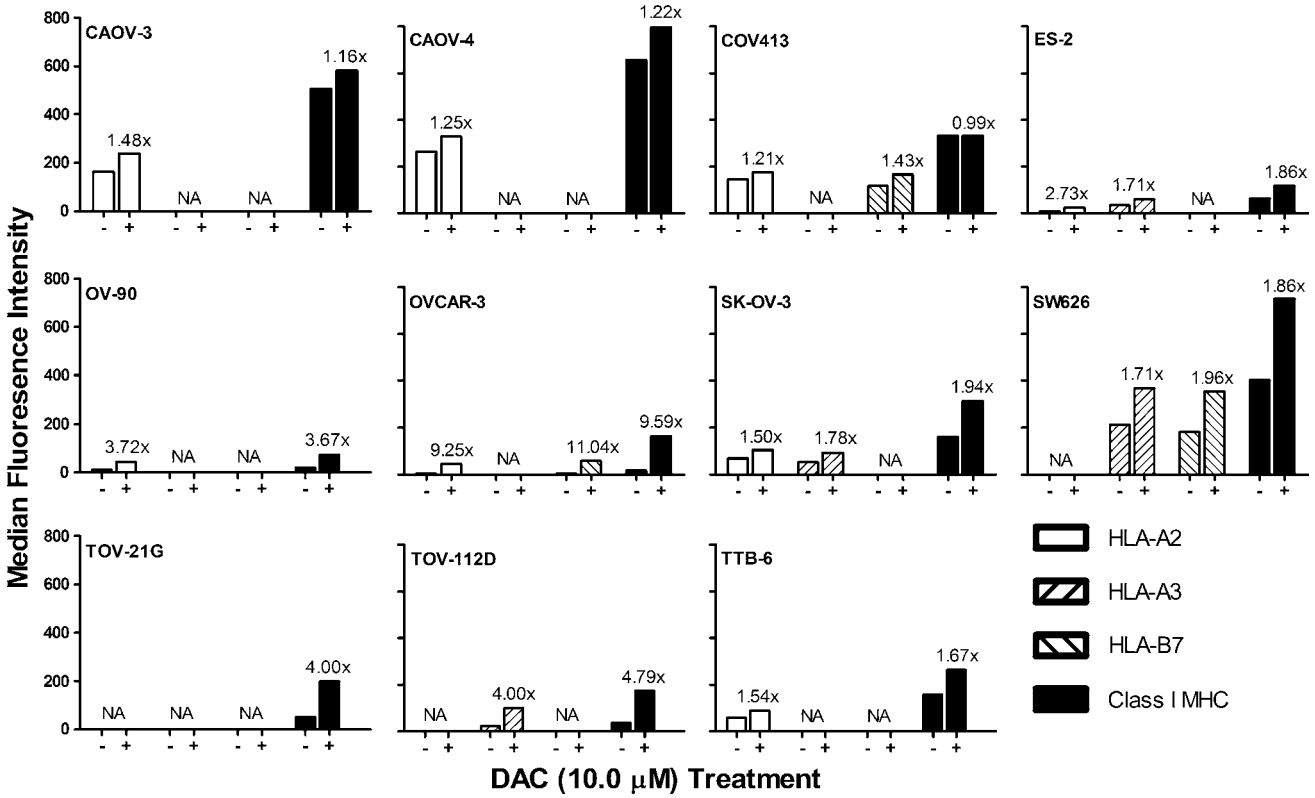

Fig. 7 The effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on class I MHC protein expression. Eleven ovarian cancer cell lines were incubated for 7 days in the presence (+) or absence (−) of 10 μM DAC and were then harvested. Class I MHC protein expression levels were determined by flow cytometry using mAb CR11-351 to detect HLA-A2, mAb GAP-A3 to detect HLA-A3, mAb ME1-1.2 to detect HLA-B7, and mAb W6/32 to detect all class I MHC molecules. The data represent one of two independent experiments

(USB technical data sheet). mAb 3F257 was used to ask if MAGE-A1 protein expression increased in parallel with CTA gene expression following DAC treatment. Although MAGE-A1 protein expression increased by 1.9- to 4.0-fold in five of the 11 cell lines tested (Fig. 6), the magnitude of the increase is relatively small in comparison to the corresponding increase in gene expression (Table 1, Supplementary Fig. 3). This would suggest that other factors limit the translation of MAGE-A1 mRNA, such that there is no linear relationship between mRNA and protein expression. Alternatively, increased levels of the protein may be associated with an increased rate of degradation of the protein.

The treatment of ovarian cancer cell lines with DAC leads to an increase in the levels of class I MHC protein expression (Fig. 7), similar in magnitude to that observed when melanoma cell lines are treated with DAC [10, 19, 49]. When cell lines already express class I MHC molecules at intermediate to high levels, the amount of increase if any, is less than twofold (e.g., CAOV3, CAOV4, and SW626). This is in accord with the fact that when gene expression levels are already high it is likely that the promoter region of the gene already exists in an unmethylated state and treatment with DAC would not be expected to have a large effect on expression. When cell lines naturally express low levels of class I MHC molecules, treatment with DAC can increase expression by four- to tenfold (see OVCAR-3, TOV-21G, and TOV-112D). In some poorly expressing cell lines such as ES-2 and SK-OV-3, however, class I MHC protein expression is increased by less than twofold. That the expression levels in these cell lines are not increased to the high levels naturally expressed in other cell lines likely reflects the fact that the low expression levels are only partially regulated, or are not regulated at all, by a methylation dependent mechanism. One or more genetic defects, either in the class I MHC genes themselves or in one of the ancillary genes (β_2-microglobulin, proteasomes, TAP, etc.) required for class I MHC protein expression could also account for the low expression levels of the class I MHC proteins [48] .

From an immunological perspective the importance of CTAs is that they are recognized as antigens by both antibodies and T cells, with the latter requiring that the CTA first be processed into peptides, which then bind to class I MHC molecules for presentation to $CD8^+$ T cells. From this perspective, a change in CTA and/or class I MHC molecule expression in response to DAC treatment is functionally significant if it alters the ability of the treated cell line to be recognized by antigen-specific $CD8^+$ T cells. As measured

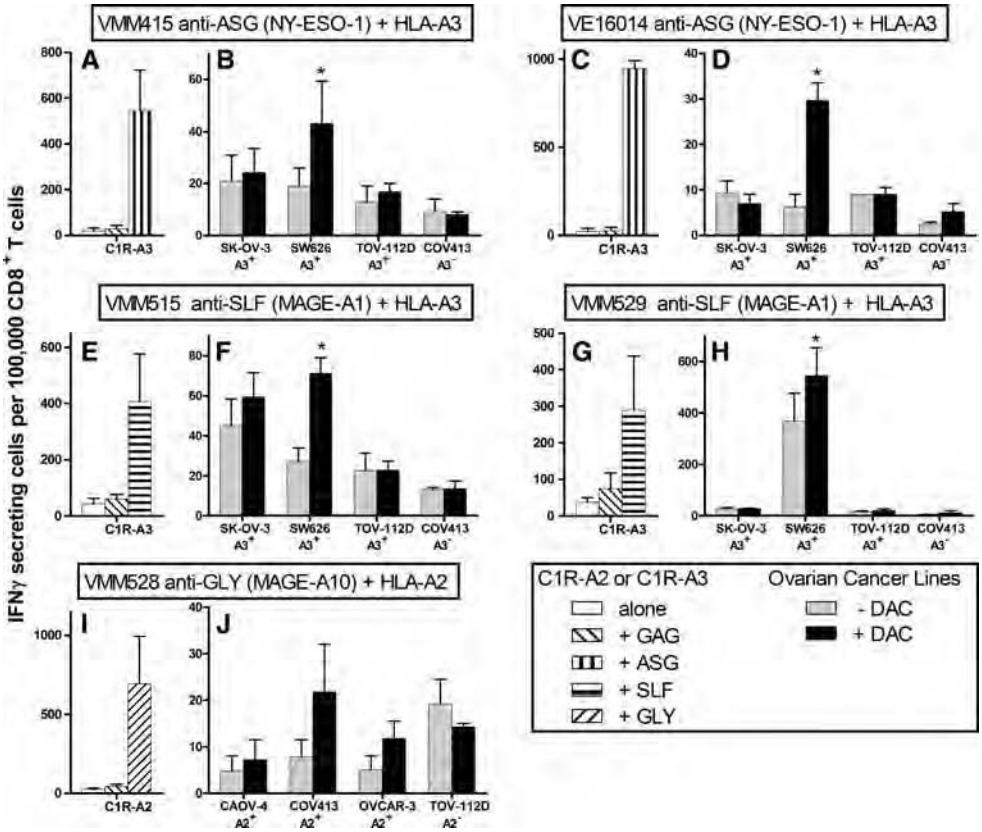

Fig. 8 The effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on recognition by antigen-specific $CD8^+$ T lymphocytes. The indicated ovarian cancer cell lines were incubated for 7 days in the presence or absence of 10 μM DAC and were then harvested. The cells were then used as stimulator cells in an interferon-γ ELISpot assay. $CD8^+$ T lymphocytes reactive to the NY-ESO-1-dervied peptide ASG presented in association with HLA-A3 (**a**–**d**), for the MAGE-A1-derived peptide SLF presented in association with HLA-A3 (**e**–**h**), and the MAGE-A10-derived peptide GLY presented in association with HLA-A2 (**i**, **j**) were used. C1R-A2 and C1R-A3 cells pulsed with the relevant peptide served as a positive control, while C1R-A2 and C1R-A3, either unpulsed or pulsed with a control peptide (GAG), served as negative controls. Each panel of ovarian cancer cells used as stimulators included three lines that were HLA-matched for the relevant class I MHC molecule and one line that was HLA-unmatched for the relevant class I MHC molecule. The data are presented as the mean ± SEM for two independent experiments

by interferon-γ release in ELISpot assays, treatment of SW626 with DAC resulted in increased recognition by NY-ESO-1/HLA-A3-reactive and MAGE-A1/HLA-A3-reactive T cells. A similar increase was not observed with cell lines SK-OV-3 and TOV-112D. These lines are similar in that all of them express intermediate basal levels of the MAGE-A1 gene and high, DAC-stimulated levels of the gene. Conversely, the basal level of expression of HLA-A3 by SW626 cells exceeds the DAC-stimulated levels of HLA-A3 expressed by the cell lines SK-OV-3 and TOV-112D. Thus, the increase in CTA expression may not manifest itself in increased recognition by $CD8^+$ T cells unless there is a certain minimum number of class I MHC molecules available to present the antigen. Similar experiments with $CD8^+$ T cells specific for a MAGE-A10-derived peptide seen in association with HLA-A2 did not result in a statistically significant increase in recognition, although the cell line COV413 had a trend toward increased recognition. It is difficult to compare these results to published results as different methods have been used to measure the T cell response including total interferon-γ release [49, 57], cytotoxicity measured by ^{51}Cr-release [8, 19, 56], and interferon-γ ELISpot [19]. The most comparable study using interferon-γ ELISpot [19] showed a doubling in the number of spots obtained, a result that is similar in magnitude to that observed here.

There are two potential limitations to the clinical use of DNA methylation inhibitors for the purposes of increasing CTA and class I MHC molecule expression. First, promoter methylation is one of many mechanisms that regulates the gene expression and methylation status does not always correlate with gene expression. For example, there is no correlation between methylation status and HLA-G expression in ovarian cancer tumors and normal ovarian epithelial cells [38], and the degree of methylation status of the MAGE-A1 gene does not show a strong correlation with gene expression [54]. Likewise, demethylation of the promoter region of class I MHC genes will not overcome the

lack of expression due to defects in the genes coding for class I MHC molecules, β_2-microglobulin, proteasomes, and TAP [48]. Thus, this approach can be useful in driving a high expression level of CTA and class I MHC molecules on some, but not all tumor cells. Second, DNA methylation inhibitors are not specific for tumor cells but have the potential to act on all cycling cells in the body. Consistent with this, in vitro DAC treatment has been shown to upregulate CTA expression of normal cells in some [12, 35], but not all [56] studies. The de novo expression of CTA by normal tissues raises the risk that CTA-specific T cells will interact with normal cells as well as tumors. Accordingly, pre-clinical studies will need to assess the degree to which epigenetic therapies increase CTA expression by normal cells, whether different dosing strategies can be used to p`entially enhance CTA expression by tumors and not normal tissue, and to determine if induced CTA expression levels on normal tissue are functionally significant from the perspective of recognition by T lymphocytes.

The results presented here demonstrate that the treatment of ovarian cancer cells with DAC can lead to a large increase in the expression of multiple CTA genes, a modest increase in the expression of class I MHC proteins, and enhanced recognition of the treated cells by antigen-specific $CD8^+$ T cells. Thus, there is a rational basis for treating ovarian cancer with combined DAC therapy and vaccine therapy designed to stimulate a T cell-mediated immune response.

Acknowledgments This work was supported by grant W81XWH-05-1-0012 from the United States Department of Defense to K. T. Hogan. The authors wish to thank Dr. Craig L. Slingluff Jr. for reviewing the manuscript and the members of the UVA Human Immune Therapy Center core laboratory for performing the ELISpot analyses.

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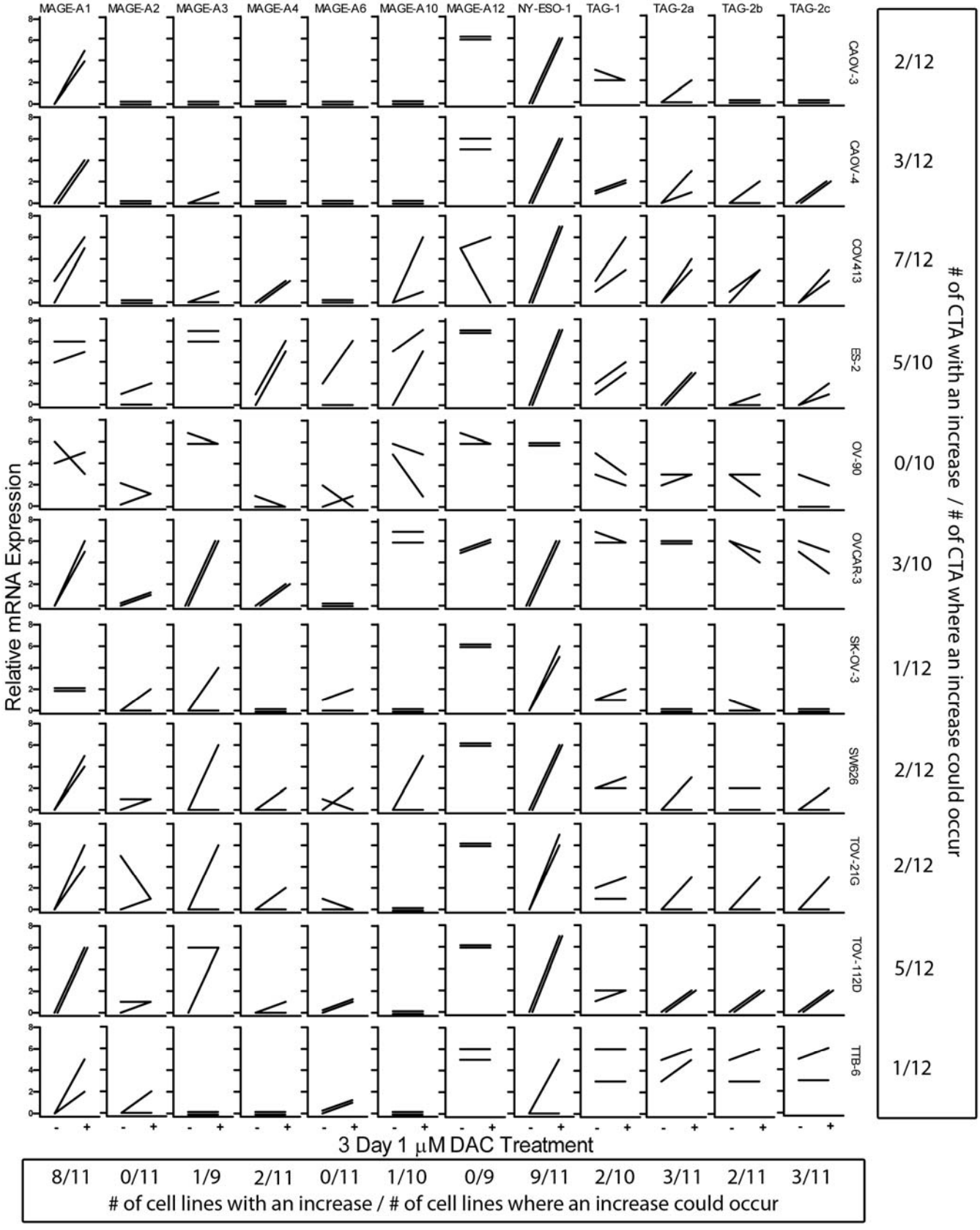
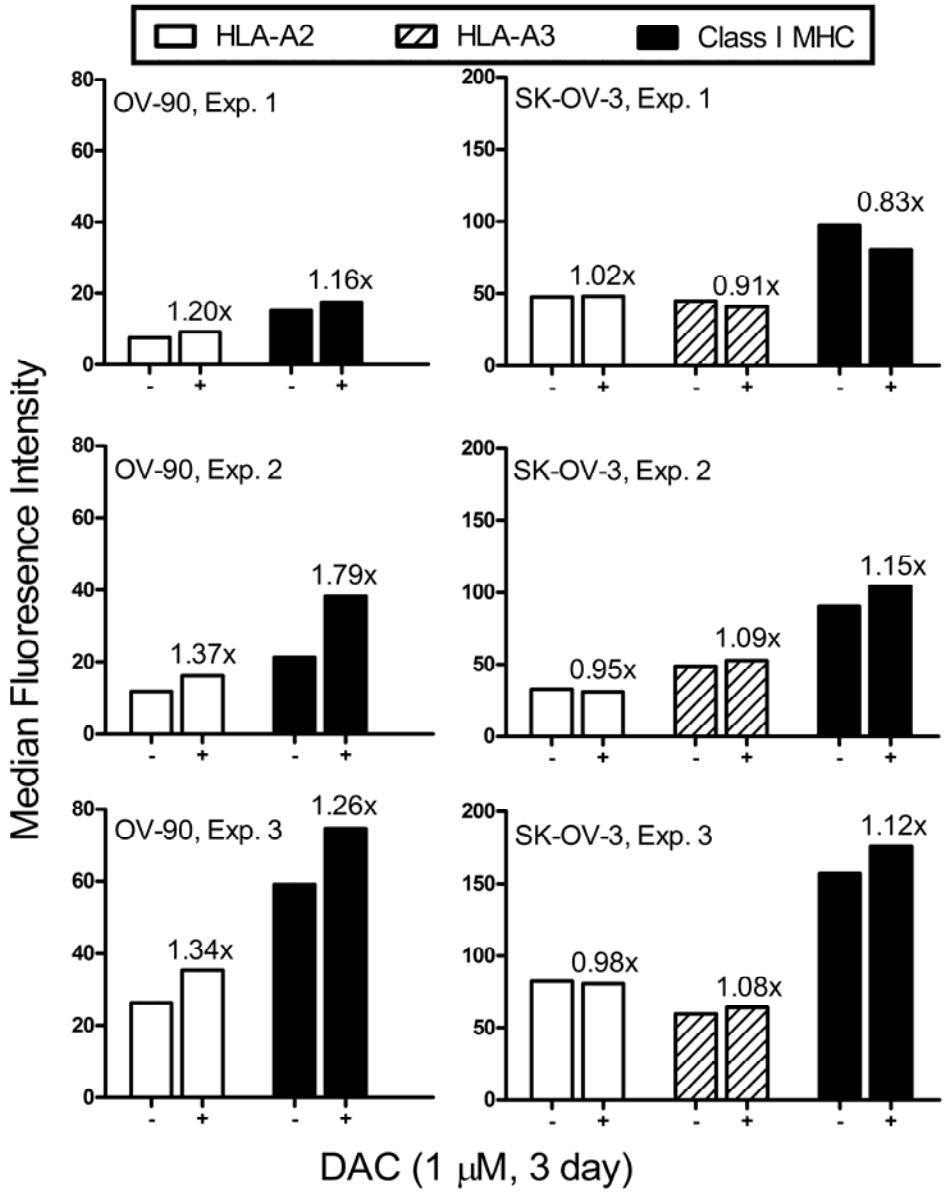
APPENDIX 3

Supplemental Fig. 1. The effect of treating ovarian cancer cell lines with 1 μM DAC for 3 days on CTA gene expression. Eleven ovarian cancer cell lines were incubated for 3 days in the presence or absence of 1 μM DAC, harvested, and cDNA prepared. CTA gene expression was determined by PCR as indicated in the Materials and Methods. Each line on the graph represents an individual experiment wherein CTA gene expression was obtained in the absence (-) or presence (+) of DAC treatment. An increase in expression following DAC treatment was defined as an increase in expression in each individual experiment and an increase of 2 or more units on the relative expression scale in at least two of the experiments. When a particular CTA/cell line pair could not show an increase because the untreated cells in at least one experiment had a relative expression level of 7 or 8 on a scale of 8, these data points were excluded from the denominator.



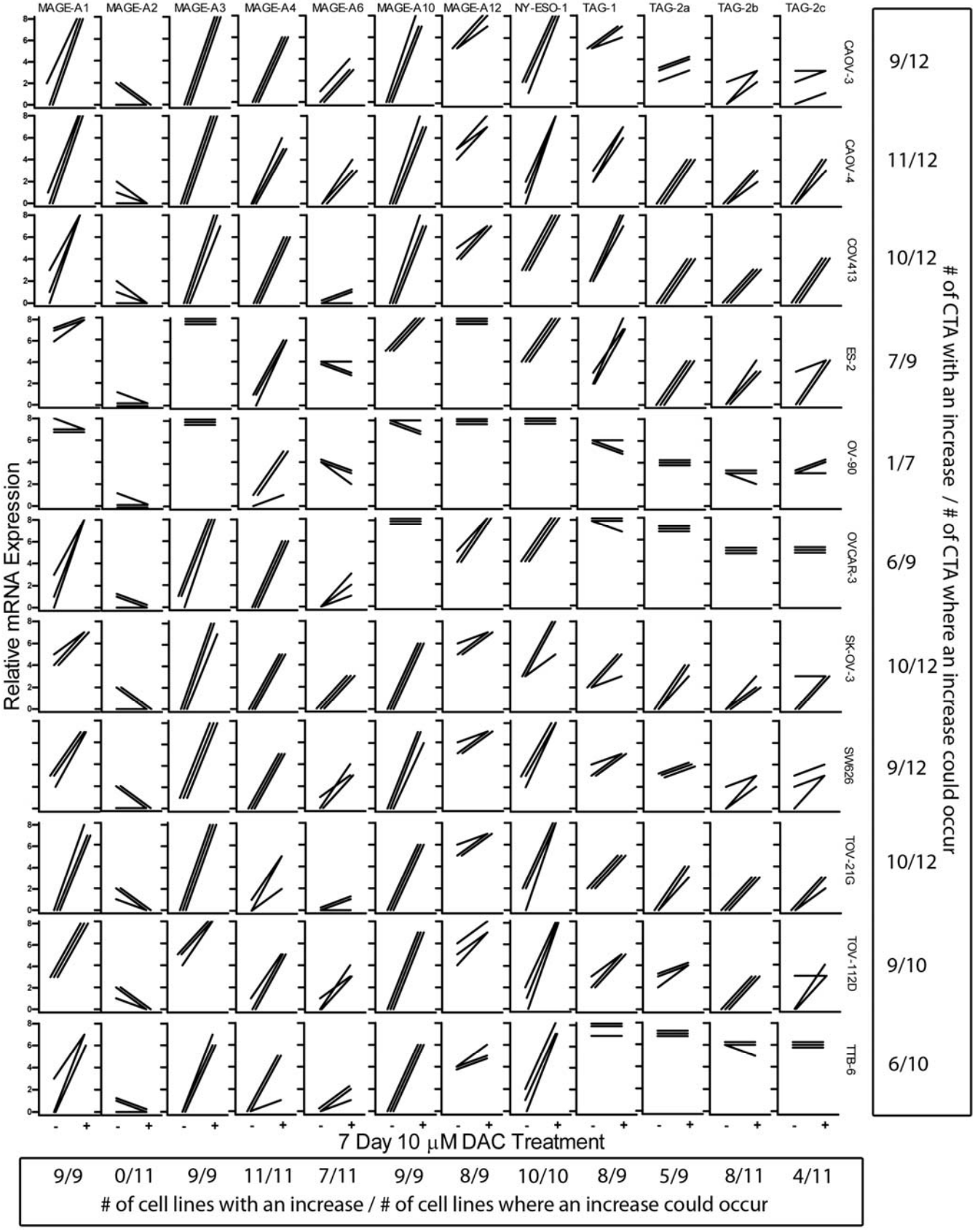
APPENDIX 3

Supplemental Fig. 2. The effect of treating ovarian cancer cell lines with 1.0 μM DAC for 3 days on class I MHC protein expression. The ovarian cancer cell lines were incubated for 3 days in the presence (+) or absence (-) of 1.0 μM DAC, harvested, and tested for class I MHC protein expression by flow cytometry. mAb CR11-351 was used to detect HLA-A2, mAb GAP-A3 was used to detect HLA-A3, and mAb W6/32 was used to detect all class I MHC molecules. The number above the (+) column indicates fold-change relative to the paired (-) column. The results from one of four independent experiments are shown.



APPENDIX 3

Supplemental Fig. 3. The effect of treating ovarian cancer cell lines with 10 μM DAC for 7 days on CTA gene expression. Eleven ovarian cancer cell lines were incubated for 7 days in the presence (+) or absence (-) of 10 μM DAC, harvested, and cDNA prepared. CTA gene expression was determined by PCR as indicated in the Materials and Methods. An increase in expression following DAC treatment was considered to have occurred if in each individual experiment there was an increase in expression, and if in at least two of the experiments the increase was 2 or more units on the relative expression scale. The results from three independent experiments are shown.



Identification of Cytotoxic T Lymphocyte Epitopes Derived From the Cancer/Testis Antigen, TAG

Sara J. Adair, Tiffany M. Carr, *Kevin T. Hogan. Department of Surgery, University of Virginia, Charlottesville, VA.*

Cancer/testis antigens are excellent candidates for inclusion in cancer vaccines as they are naturally immunogenic, are expressed in a high percentage of tumors of diverse histological origin, and their expression in normal tissue is limited to the testis and placenta. We have recently identified a new cancer/testis antigen termed TAG which is expressed as multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) at the mRNA level. TAG is known to be immunogenic as it gives rise to an HLA-A3-restricted epitope (RLSNRLLLR) that is recognized by cytotoxic T lymphocytes (CTL) that naturally occur in a melanoma patient. To determine if TAG gives rise to additional epitopes recognized by CTL we have used two predictive algorithms (SYFPEITHI [www.syfpeithi.de] and Parker [bimas.cit.nih.gov/molbio/hla_bind] to identify those peptides that have a high predicted binding affinity for HLA-A1, -A2, -A3, -B7, -B8, and -B44. The top three to five predicted binders for each algorithm/HLA combination have been synthesized and are being tested for their ability to elicit tumor reactive CTL from healthy blood donors. Donor lymphocytes are initially stimulated with peptide-pulsed, autologous, mature dendritic cells (mDC), and are then restimulated thereafter on a weekly basis with peptide-pulsed stimulator cells (autologous mDC, autologous peripheral blood mononuclear cells, or class I MHC-matched allogeneic B-LCL). Following a total of four stimulations, the cultures are tested for reactivity against peptide-pulsed, class I MHC-matched target cells. Using this protocol, CTL responses have developed in response to SLGWLFLLL peptide in association with HLA-A2 and LSRLSNRLL peptide in association with HLA-B8, but not to SRDPPASAS peptide in association with HLA-A1, LLLLNSTTK peptide in association with HLA-A3, or LPASTLSRL peptide in association with HLA-B7. SLGWLFLLL reactive CTL also lyse the melanoma line DM6 (HLA-$A2^+$, TAG^+) indicating that the SLGWLFLLL peptide is naturally processed. Experiments are in progress to further characterize the CTL response to SLGWLFLLL and LSRLSNRLL, and to determine the immunogenicity of additional TAG-derived peptides. (Supported by DOD W81XWH-05-1-0012 and NIH/NCI CA90815)

Immunological Characterization of Eleven Human Ovarian Cancer Cell Lines

Kevin T. Hogan, Tiffany M. Carr, Sara J. Adair, Mitsú J. Fink. *University of Virginia, Charlottesville, VA.*

The study of the cellular immune response to ovarian cancer requires the use of well-characterized tumor cell lines. To develop such a panel of cell lines, 11 ovarian cancer cell lines were characterized for the expression of epithelial cell markers, class I and class II major histocompatibility complex (MHC)-encoded molecules, 15 tumor antigens (MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, TAG-2c, Her-2/neu, folate-binding protein, and carcinoembryonic antigen), and immunosuppressive cytokines [transforming growth factor β (TGF-β) and IL-10]. Each of the ovarian cancer cell lines expresses cytokeratins, although each cell line does not express the same cytokeratins. One of the lines expresses CD90 which is associated with a fibroblast lineage. Each of the cell lines expresses low to moderate amounts of class I MHC molecules, and several of them express low to moderate amounts of class II MHC molecules. Treatment of the cell lines with 1 μM 5′-aza-2′-dexoycytidine (DAC) for 72 hours, a treatment that can up-regulate the expression of class I and II MHC molecules in other malignancies, did not reproducibly up-regulate the expression of these molecules on the 11 ovarian cancer cell lines. Using a combination of PCR and flow cytometry, it was determined that each cell line expressed between 6 and 13 of 15 antigens tested. Although in vitro treatment of other malignancies with DAC has been shown to up-regulate the expression of a variety of cancer/testis antigens, only the expression of NY-ESO-1 was reproducibly up-regulated in a majority of the cell lines tested. TGF-β1 was produced by 3 of the cell lines, TGF-β2 was produced by all of the cell lines, with 4 of the cell lines producing large amounts of the latent form of the molecule, and little to no TGF-β3 was produced by any of the cell lines. SW626 was the only cell line that produced IL-10. These results demonstrate that each of the 11 ovarian cancer lines is characterized by a unique expression pattern of epithelial/fibroblast markers, MHC molecules, tumor antigens, and immunosuppressive cytokines. The results further demonstrate that unlike other malignancies, treatment of ovarian cancer cells with DAC may not lead to an increase in class I MHC expression, class II MHC expression, and the expression of many cancer/testis antigens. This knowledge increases the utility of these cell lines for use in antigen identification experiments and suggests that therapeutic treatment of ovarian cancer patients with DAC may not be useful in enhancing the expression of antigens targeted in vaccines designed to stimulate a cytotoxic T lymphocyte response.